Genetic epidemiology of postmenopausal osteoporosis.

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GENETIC EPIDEMIOLOGY OF POSTMENOPAUSAL OSTEOPOROSIS

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ABSTRACT

Osteoporosis is a common condition affecting up to one in three women. This thesis has examined the genetic epidemiology of this disease, with analysis of postmenopausal twin pairs and unrelated women from the UK general population.

Classical twin analysis in 87 monozygous and 95 dizygous (DZ) twin pairs demonstrated significant heritability for bone mineral density (BMD) at multiple skeletal sites, suggesting that between 60-85% of the population variance in bone mass was attributable to genetic factors. Within the DZ twins, a biallelic *TaqI* polymorphism of the vitamin D receptor (VDR) gene was found to be significantly associated with BMD with additional suggestive evidence for linkage. In this group, however, a *FokI* polymorphism mapping to the 5' region of the gene was neither associated nor linked with bone mass.

Analysis of 1,003 unrelated women also demonstrated a familial tendency to osteoporosis and fracture risk. Women with a positive family history of osteoporotic fracture in a female first-degree relative had reduced bone density at both spine and hip when compared to women with a negative history. Risk of fracture was increased three-fold in those with a positive family history, with strong evidence of site specificity for wrist fracture. A polymorphism of the type I collagen 1α gene was associated with reduced BMD and increased fracture risk, whilst an interleukin-1 receptor gene polymorphism was found to be associated with rates of menopausal bone loss. Negative results were observed with the oestrogen and VDR gene polymorphisms, although the VDR *TaqI* polymorphism was independently associated with risk of knee and spine osteoarthritis.

In conclusion, these results demonstrate that osteoporosis is under significant genetic control with a complex inheritance pattern. Several genes appear to act on differing bone-related traits with preliminary evidence for interaction between genes and with other co-morbid disease states.

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DECLARATION

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

In this study I was responsible for the following:

- Planning of study design
- DNA extraction
- Genotype analysis
- Statistical analysis of all data

LIST OF ABBREVIATIONS

μ	Mean
μg	Microgramme or 10^{-6} g or one-millionth of 1 g
μl	Microlitre or 10^{-6} l or one-millionth of 1 litre
μM	Micromole or 10^{-6} mole or one-millionth of mole
1,25-$(OH)_2D_3$	1,25- dihydroxyvitamin D_3
A	Adenosine
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ASP	Affected sib pair
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
bp	Base pair
BUA	Broadband ultrasound attenuation
C	Cytosine
cDNA	Cloned DNA
CI	Confidence interval
cm	Centimetre or 10^{-2} of a metre
cM	Centimorgan or 10^{-2} of a Morgan
COL2A1	Type 2 collagen α1
COLIA1	Type 1 collagen α1
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate

DPA	Dual photon xray absorptiometry
dsDNA	Double stranded DNA
DXA	Dual energy xray absorptiometry
DZ	Dizygous
ER	Oestrogen receptor
EVOS	European Vertebral Osteoporosis Study
FH	Family history
FSH	Follicle stimulating hormone
g	Gramme or 10^{-3} of a kilogramme
G	Guanine
h^2	Heritability
HAL	Hip axis length
HRT	Hormone replacement therapy
hVDR	Human vitamin D receptor
HWE	Hardy Weinberg equilibrium
IBD	Identical by descent
IBS	Identical by state
ICTP	Carboxy-terminal telopeptide of type I collagen
IL-1	Interleukin 1
IL-1ra	Interleukin 1 receptor antagonist protein
IL-1RN	Interleukin 1 receptor antagonist gene
kb	Kilobases
kg	Kilogramme
l	Litre
LD	Linkage disequilibrium

LH	Leutinising hormone
M	Molecular weight in grammes
m	metre
MEDOS	Mediterranean Osteoporosis Study
mg	Milligrammes or 10^{-3} g or one-thousandth of 1 g
ml	Millilitre or 10^{-3} l or one-thousandth of 1 l
mM	Millimole or 10^{-3} M or one-thousandth of 1 M
mnths	Months
mRNA	Messenger RNA
MZ	Monozygous
ng	Nanogramme or 10^{-9} g or one-thousand-millionth of 1 g
NPV	Negative predictive value
OA	Osteoarthritis
OC	Osteocalcin
OI	Osteogenesis imperfecta
OR	Odds ratio
PCR	Polymerase chain reaction
PDDR	Psuedodeficiency vitamin D rickets
PICP	C-propeptide of type I collagen
pmol	Picomole or 10^{-12} M or one-million-millionth of 1 M
PPV	Positive predictive value
PTH	Parathyroid hormone
QUS	Quantitative ultrasound
r	Correlation coefficient
rDZ	Intra-class correlation for non-identical twin pairs

RFLP	Restriction fragment length polymorphisms
rMZ	Intra-class correlation for identical twin pairs
SD	Standard deviation
SE	Standard error
SOF	Study of Osteoporotic Fractures
SPA	Single photon xray absorptiometry
T	Thymidine
UK	United Kingdom
US	United States
V	Variance
VDR	Vitamin D receptor
VNTR	Variable number tandem repeat
VOS	Velocity of sound
WHO	World Health Organisation
YAC	Yeast artificial chromosome
yrs	Years

PUBLICATIONS ARISING FROM THE WORK OF THIS THESIS IN PEER-REVIEWED JOURNALS

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DEDICATION

I am indebted to my parents for both the "nature" and the "nuture" during my informative years, thereby providing me with the opportunity to study medicine and to develop an early interest in genetics.

I would also not have been able to undertake this thesis without the love, support and encouragement of my family. This work is therefore dedicated to my wife Nikki and our three children Ellie, Matthew and Ben.

CHAPTER I

INTRODUCTION AND BACKGROUND

I.1 Summary

Osteoporosis is a common skeletal disease characterised by low bone mass. deterioration of skeletal architecture and an increased susceptibility to fragility fracture. Approximately one in three women will experience an osteoporotic fracture during their lifetime, with the male lifetime risk being 25% of that observed in females. Bone mineral density (BMD) in later life is the strongest predictor of subsequent fracture, and is a composite value determined positively by the peak BMD achieved during skeletal growth and negatively by both age- and menopause-related bone loss. This chapter examines the aetiology of osteoporosis and the epidemiological risk factors associated with low BMD and fracture risk. A comprehensive review of genetic epidemiological studies related to osteoporosis is presented, utilising data from both family- and twin-based studies. With a hypothesis that genetic factors play a central role in the determination of bone mass, a discussion is given on likely candidate genes that may play a key regulatory role. The aim of this thesis is to provide novel insight into the genetic epidemiology of osteoporosis and fracture risk in Caucasian women, and to examine the role of potential candidate loci in determining postmenopausal bone mass. Knowledge of the genetic mechanisms underlying both the attainment of peak BMD and subsequent bone loss will lead to an increased understanding of the disordered pathophysiology associated with osteoporosis and may ultimately result in improved therapeutic and diagnostic options for this common disease.

I.2 Introduction

The term "osteoporosis" entered medical terminology in France and Germany during the last century. In its initial form it implied a histological diagnosis of porous bone, although this was subsequently refined to state that although bone tissue was present in a reduced quantity it was normally mineralised (Schapira and Schapira, 1992). Clinically osteoporosis is recognised by the occurrence of characteristic fractures at thoracolumbar spine, hip and wrist.

A World Health Organisation (WHO) Study Group has recently attempted to combine both the histological and clinical definitions for osteoporosis. They state that osteoporosis is "a disease characterised by low bone mass and microarchitecture deterioration of bone tissue, leading to enhanced bone fragility and a consequent increase in fracture risk" (Consensus Development Conference, 1991). Implicit in this definition of osteoporosis is the trait of low bone mass which can now be accurately measured non-invasively. The WHO (1994) has also recently proposed at a meeting of experts an operational definition for osteoporosis with reference to the peak young normal mean value (Table I.1). This is based on the number of standard deviations a subject's BMD is above (+) or below (-) this mean peak value, and these results are expressed as a T-score. Using these definitions it has been possible to determine the population prevalence of osteoporosis at various ages, although the validity of this definition for males is still unclear (Table I.2).

Table I.1 Diagnostic classification for osteopenia and osteoporosis

WHO Disease Classification	BMD Measurement
Normal	T-score above -1
Osteopenia	T-score between -1 and -2.5
Osteoporosis	T-score below -2.5
Established Osteoporosis	T-score below -2.5 and fragility fracture

Table I.2 Prevalence of osteoporosis at spine and hip in UK women according to age (Compston et al, 1995)

	Age 50-54 yrs (%)	Age 70-74 yrs (%)
Lumbar Spine	3.5	15
Femoral Neck	2.0	20

Table I.3 Lifetime risk of common osteoporotic-related fractures in British men and women aged 50 years (Cooper 1996)

	Men (%)	Women (%)
Hip	3	14
Vertebral (spine) *	2	11
Distal forearm (wrist)	2	13

* Data for clinically diagnosed vertebral deformity

Despite these attempts to provide uniform standards for disease definition, the prevalence estimates for osteoporosis remain complicated by the population reference ranges selected to define the mean BMD for the young normal population, the site of measurement and the type of machine and software used for scanning.

Osteoporosis affects an estimated 75 million people in Europe, Japan and the United States (US) combined, including one in three postmenopausal women and most elderly people (Melton et al, 1992; Melton 1995a). Male rates of osteoporosis appear to be 25 % of that reported in women. The major health consequence of osteoporosis is that of fracture, and as stated previously the primary fracture sites are found at the long bones and the vertebrae. Current figures for the United Kingdom (UK) show that there are 60,000 hip fractures per year, 40,000 wrist (Colles) fractures and 40,000 symptomatic vertebral fractures per year (AGO Report, 1994). The lifetime risk for a 50 year old woman sustaining an osteoporotic-related fracture is estimated at 38% (Black et al 1992; Cooper 1996) (Table I.3).

Fractures of the spine and wrist are painful and can result in skeletal deformity. Hip fracture however is responsible for much of the mortality and morbidity due to osteoporosis and is a leading cause of disability in elderly people. Between 12-20 % of patients with hip fracture will die within 1 year of the event (Keene et al, 1993), and mortality increases progressively with advancing age. There is also a large degree of morbidity associated with hip fractures as the majority of those that survive are unable to perform the activities of daily living unaided and a small percentage require permanent care in an institution or nursing home. Annual health costs for the care of osteoporotic

fractures within the UK are estimated at £942 million per year (AGO Report, 1994; Dolan and Torgerson, 1998).

I.3 Epidemiology of osteoporosis

A risk factor is described as an attribute associated with an increase in the risk of the disease. Some risk factors may be measures of the basic biologic processes that cause disease whilst others may merely be markers of risk, only indirectly linked to the pathogenesis of the disease. BMD is an established determinant of bone strength, and several studies have shown that it is a strong predictor of subsequent fracture (Hui et al, 1989; Cummings et al 1990; Cummings et al 1993). Many risk factors are thought to influence osteoporotic fractures through direct effects on BMD, whilst others act through independent factors contributing to other facets of skeletal strength (i.e. architecture or quality), or by influencing the risk of falls. Many studies have identified epidemiological risk factors that are associated with osteoporosis and fracture at the population level (Cummings et al, 1995), although these risk factors appear too insensitive to act on their own as predictive tools for individual subjects (Spector et al, 1992) (Table I.4). It remains to be determined, however, whether any of these risk factors can be modified in an effective manner to significantly reduce the incidence of osteoporosis and the healthcare burden and cost associated with fracture.

Most risk factors for osteoporotic fracture fall into six main categories: age or age-related, environmental, hormonal, chronic diseases, physical characteristics of bone, and racial and genetic factors.

Table I.4 **Crude relative risks for osteoporotic hip fracture associated with epidemiological risk factors in 9516 white women (Cummings et al, 1995)**

Measurement (comparison or unit)	Relative risk (95% confidence intervals)
Age (per 5 yrs)	1.4 (1.2-1.6)
History of maternal hip fracture (vs none)	1.8 (1.2-2.7)
Increase in weight since age 25 (per 20%)	0.8 (0.6-0.9)
Height at age 25 (per 6 cm)	1.3 (1.1-1.5)
Self-rated health (per 1-point decrease) #	1.6 (1.2-2.1)
Previous hyperthyroidism (vs none)	1.7 (1.2-2.5)
Current use of benzodiazepines (vs no current use)	1.6 (1.1-2.4)
Current use of anticonvulsant drugs (vs no current use)	2.0 (0.8-4.9)
Current caffeine intake (per 190mg/day)	1.2 (1.0-1.5)
Walking for exercise (vs not walking for exercise)	0.7 (0.5-1.0)
On feet ≤ 4hr/day (vs > 4 hr/day)	1.7 (1.2-2.4)
Inability to rise from chair (vs ability)	1.7 (1.1-2.7)
Lowest quartile for distal depth perception (vs other three)	1.4 (1.0-1.9)
Low-frequency contrast sensitivity (per 1 SD decrease)	1.2 (1.0-1.5)
Resting pulse > 80 beats/min (vs ≤ 80 beats/min)	1.7 (1.2-2.4)
Any fracture since age of 50 (vs none)	1.5 (1.1-2.0)
Calcaneal BMD (per 1 SD decrease)	1.6 (1.3-1.9)

Health was rated as poor (1 point), fair (2 points), or good to excellent (3 points)

I.3.1 Risk factors - Age

As previously demonstrated in Table I.2, the prevalence of osteoporosis defined using BMD criteria increases with age. During childhood and adolescence there is rapid linear and appositional skeletal growth, the former reaching a maximum in the latter half of the second decade of life. Peak bone mass is attained during the third decade of life, with age-related bone loss commencing during the fourth decade. This loss continues throughout life and is accelerated in women at the time of the menopause (Figure I.1). Overall it is estimated that approximately 50% of trabecular bone and 35% of cortical bone mass are lost over a lifetime in women, with losses in men approximately two-thirds of these amounts.

The common osteoporotic fractures also increase with age and show characteristic incidence profiles that are site specific (Figure I.2). Hip fractures increase exponentially after the age of 70 years, with the average age for a hip fracture in the UK being 79 years. This is due to both age-related reductions in bone strength and to an age-related increase in the risk of falling. Winner et al (1989) demonstrated that whereas one in three women aged 80-84 years had fallen in the previous year, this figure had risen to almost 50% in women aged 85 years and over.

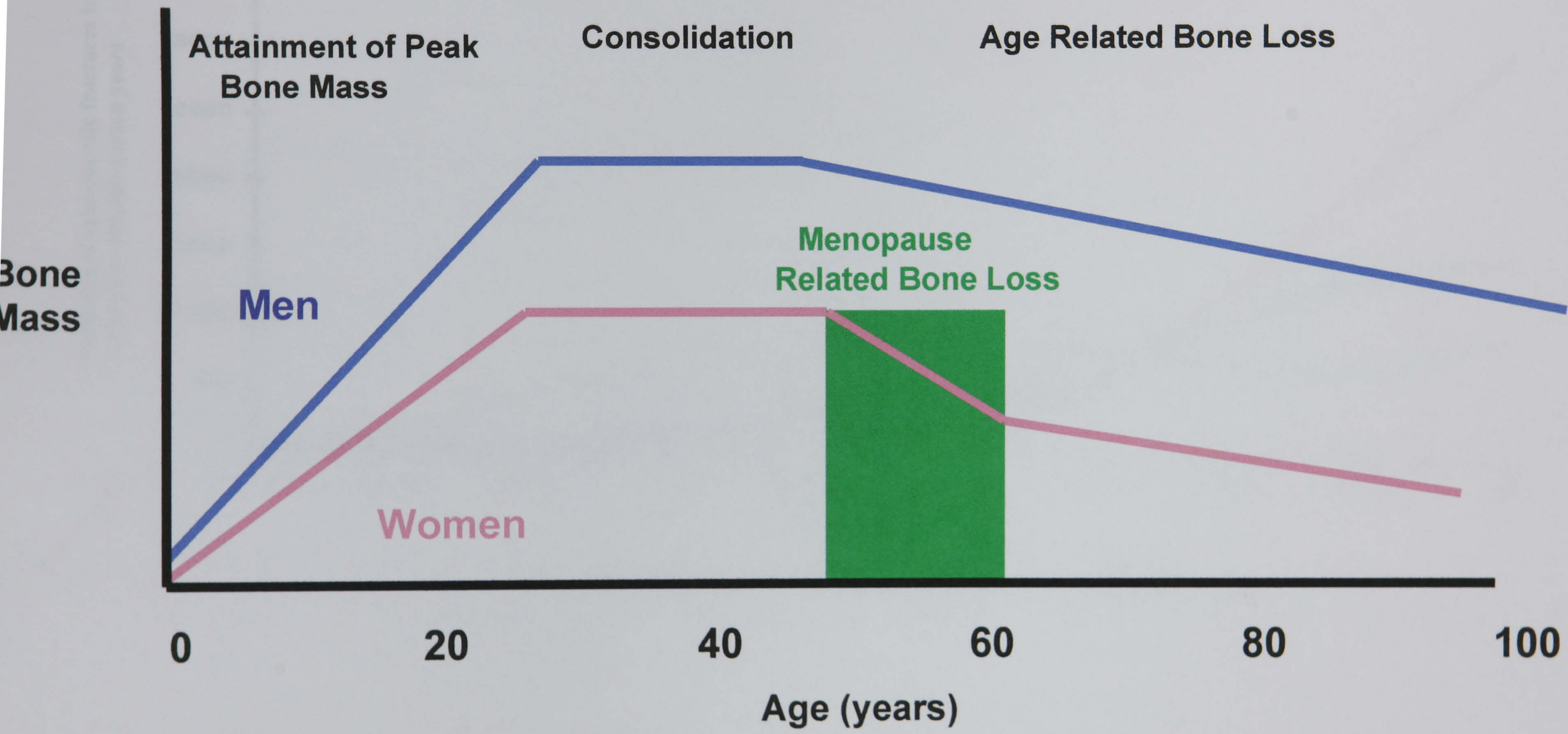


Figure I.1 Age-related changes of BMD in men and women

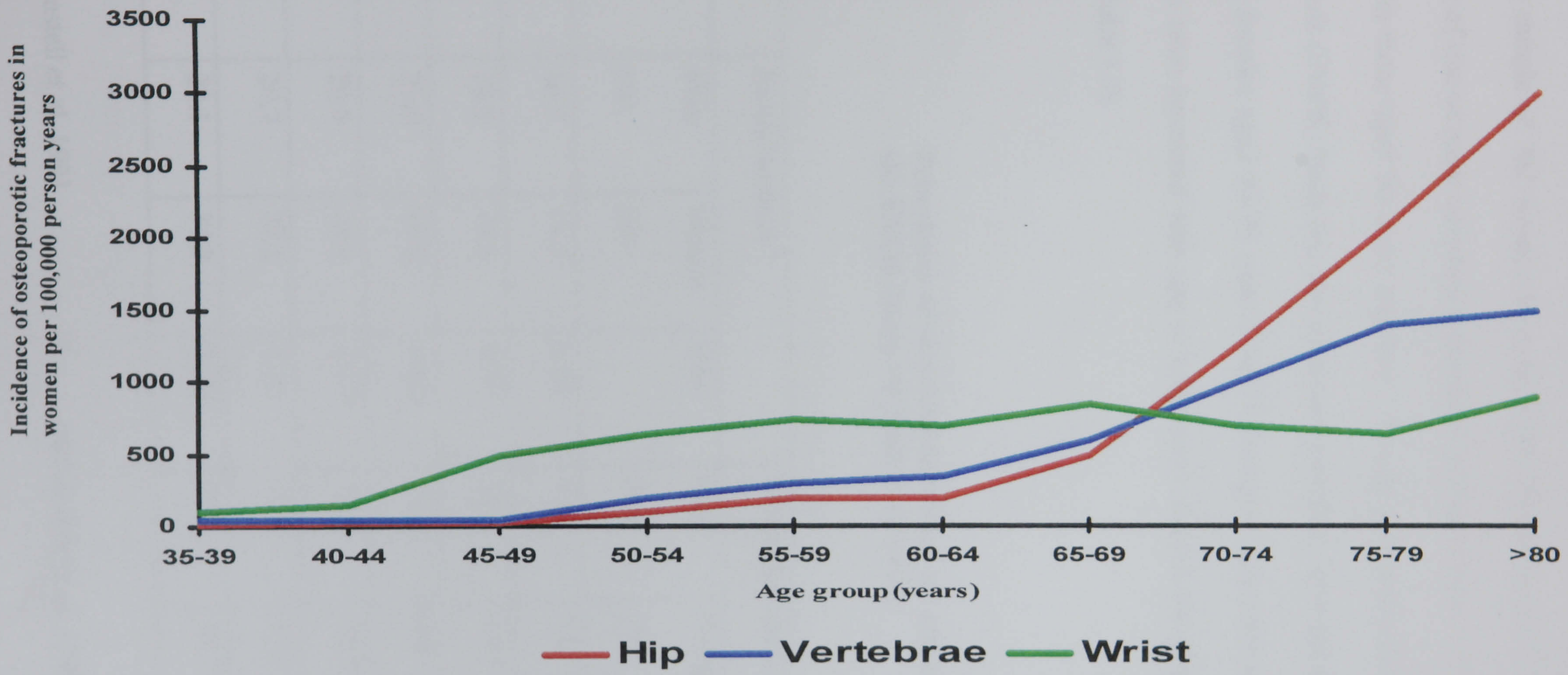


Figure I.2 Incidence of osteoporotic fractures with age

Vertebral fractures are commonly asymptomatic and prevalence estimates can only be obtained by radiological survey of the thoracolumbar spine although there remains a lack of a universally accepted definition for vertebral deformity. In a population sample of 762 women from the USA Melton et al (1993) showed that the prevalence of one or more vertebral deformities increased from 7.6% at age 50-54 years to 64.3% in those aged 90 years and over. Recent data from the European Vertebral Osteoporosis (EVOS) Study has also examined prevalent vertebral deformities in 15,570 males and females aged 50-79 years from 19 European countries (O'Neill et al, 1996). Prevalence rates increased with age in both sexes, though the gradient was steeper in women (Table I.5).

Table I.5 Prevalence of vertebral deformity by age and sex group in the EVOS Study (O'Neill et al, 1996)

	Eastell method *			McCloskey method #		
Age (yrs)	Male (%)	Female (%)	F:M	Male (%)	Female (%)	F:M
50-54	16.5	11.5	0.70	9.9	5.0	0.51
55-60	18.5	14.6	0.79	11.4	7.6	0.67
60-64	21.0	16.8	0.80	12.8	9.9	0.77
65-69	20.9	23.5	1.12	12.1	13.4	1.11
70-75	20.7	27.2	1.31	13.2	17.0	1.29
75-79	29.1	34.8	1.20	18.1	24.7	1.36

* Eastell et al, 1991 # McCloskey et al, 1993

Distal forearm fractures display a different incidence pattern to that of the other osteoporotic fractures. The incidence rates for wrist fractures increase linearly from age 40-65 years and then plateau in women, whereas in males the incidence remains at a constant low rate between 20 and 80 years.

I.3.2 Risk factors - Hormonal

I.3.2.1 Oestrogen

Osteoporosis occurs more commonly in women than in men, with female fracture rates increasing markedly after the menopause. These differences probably occur because of sex differences in peak BMD and because of women experiencing accelerated bone loss at the time of the menopause. This indicates the importance of oestrogen in the determination and maintenance of BMD in women, although the importance of this hormone on the male skeleton has also been demonstrated by recent case reports (Smith et al, 1994; Morishima et al, 1995; Bilezikian et al, 1998).

The association between osteoporosis and oestrogen was first described in 1941 when it was noted that 40 of 42 women with osteoporotic fractures were postmenopausal (Albright et al, 1941). Subsequently the associations between oopherectomy and accelerated bone loss (Meema et al, 1965; Nordin et al, 1968). and the beneficial effects of oestrogen were observed (Meema et al, 1975; Lindsay et al, 1980). Bone mass has been positively associated with parity (Alderman et al, 1986) and

use of the oral contraceptive (Lindsay et al, 1986), but results have not been consistent between studies. Hypo-oestrogenic states that occur in anorexia nervosa and athletic amenorrhoea are also associated with low bone mass and increased fracture risk, although the pathogenic role of oestrogen deficiency has not been definitely established in these conditions. At the onset of the menopause there is an increase in bone turnover that results in net bone loss (Stepan et al, 1987). Bone turnover can be assessed indirectly by measurement of biochemical markers of bone formation and resorption found in serum and urine, and studies show that treatment with hormone replacement therapy (HRT) causes these markers to return to premenopausal levels (Johansen et al, 1988; Uebelhart et al, 1991).

I.3.2.2 Testosterone

Testosterone deficiency has been reported to occur in up to 30% of men with spinal osteoporosis (Jackson et al, 1987) and is a significant risk factor for hip fracture in elderly males (Jackson et al, 1992). Androgen receptors have been demonstrated in vitro in human osteoblasts and osteoblast-like cells, as well as in bone marrow stromal cells and osteoclast-like multinucleated cells (Vanderscheueren and Bouillon, 1995). The recent findings of low BMD in men with mutations in either oestrogen receptor (ER) or aromatase genes, suggests that local aromatisation of testosterone to oestradiol with direct oestrogen action on bone via its receptor may also be necessary for normal male bone homeostasis (Smith et al, 1994; Morishima et al, 1995; Bilezikian et al, 1998).

I.3.2.3 Thyroxine

Bone mass is reduced in patients with thyrotoxicosis, with the detrimental effects of thyroid hormones on the skeleton being more marked in women compared to men (Rosen et al, 1992; Daimond et al, 1994). The decrease in BMD associated with thyrotoxicosis is reversible with effective treatment, although a previous history of thyroid disease is still associated with increased hip fracture risk in elderly women (Cummings et al, 1995). Thyroid hormones increase bone remodelling and directly stimulate osteoblast production of markers such as alkaline phosphatase (ALP) and osteocalcin (OC) (Mosekilde et al, 1990; Sato et al, 1987; Rizzoli et al, 1986). Despite this increased osteoblastic activity, the enhanced bone formation cannot compensate for the thyroid-induced increase in bone resorption

I.3.2.4 Parathyroid hormone

Parathyroid hormone (PTH) regulates the levels of calcium and phosphate in blood by modulating the activity of specific cells in bone and kidney. Patients with hyperparathyroidism show reduced BMD primarily at cortical but not trabecular bone sites. Past history of hyperparathyroidism has also been shown to be a risk factor for hip fracture in the elderly (Cummings et al, 1995). PTH stimulates bone resorption, although this effect is not a consequence of direct action of PTH on the osteoclast as it does not respond to this hormone in isolation. This suggests that the presence of osteoblasts or osteoblast-derived factors is necessary for PTH-induced resorption. PTH

also has complex effects on bone formation, being able to both stimulate and inhibit bone collagen and matrix synthesis (Canalis et al, 1989; Canalis et al, 1994).

I.3.3 Risk factors - Environmental

The incidence of wrist and hip fractures exhibits a marked seasonal variation. The variation for wrist fractures is seen in Northern Europe but is not apparent in Southern European countries, and is clearly associated with an increased risk of falls in the winter months due to icy conditions. The seasonality in hip fractures is observed world-wide in both men and women, although the majority of hip fractures occur indoors and are not directly related to slipping on icy surfaces. They may therefore be a consequence of either a cold induced reduction in neuromuscular response to a falls or to reduced vitamin D levels due to low sunlight exposure in the winter months.

There is wide variation in the incidence of hip fractures within populations of a given race and gender. The Mediterranean Osteoporosis Study (MEDOS) suggested that hip fracture rates differed both between and within countries, with an 11-fold variation (Elffors et al, 1994), although some of these differences are likely to be due to sampling and selection bias. In the USA, Jacobsen (1990) studied age-adjusted hip fracture rates in white women aged above 65 years from over 2,000 counties. Risk of fracture was negatively associated with increasing northern latitude, water hardness and hours of January sunlight, and positively associated with poverty levels, proportion of the land in farms, and the proportion of the population with fluoridated water. Compared to

the hip fracture data, the EVOS Study showed a much smaller geographical variation for vertebral deformity (O'Neill et al, 1996). This may reflect the additional requirement for falls in the pathogenesis of hip fracture, although it may simply be a result of a better study design with less bias.

I.3.3.1 Calcium

Calcium is a vital component of normal functioning bone and comprises about 22% of skeletal weight. At birth the skeleton contains around 25 g of calcium, rising to 1,000-1,200 g at skeletal maturity (Widdowson 1981). Infants double their skeletal mass in the first year of life, and up to 40% of adult skeletal mass is accrued during adolescence. Estimates suggest that a mean calcium intake of 175 mg/day is required during the growth years to ensure adequate skeletal development. Matkovic et al (1979) compared bone mass in two populations from Croatia that had differing dietary calcium intakes. Subjects in the high calcium intake area had greater peak BMD and maintained this benefit throughout life. In a study of calcium supplementation in pre-pubertal twins, Johnston et al (1992) demonstrated the beneficial effects of calcium on BMD in the twin receiving the supplement. Cummings and Nevitt (1997) have recently published their results from a meta-analysis of 23 epidemiological studies examining the relationship between dietary calcium intake and osteoporotic fracture. Pooled results suggest a modest 4% reduction in hip fracture risk per 300mg/day increase in dietary calcium levels.

I.3.3.2 Calcium absorption

Calcium absorption through the gastrointestinal tract is dependent on the level of dietary intake. At low dietary intakes, calcium absorption occurs mainly via a 1.25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] dependent active transport mechanism. At levels of calcium intake greater than 400 milligrammes (mg) per day, calcium is absorbed passively (Sheikh et al, 1988). Studies in older populations have shown that intestinal calcium absorption decreases with age in both males and females (Gallagher et al, 1979; Slovik et al, 1981), although some have shown conflicting results (Christiansen and Rodbro, 1984). Ageing may alter the metabolism of calcium and vitamin D in a number of ways. There is an age-related decline in dermal production of 7-dehydrocholesterol, the precursor of pre-vitamin D_3, so that synthesis of vitamin D_3 is reduced (MacLaughlin and Holick, 1985). Intake of vitamin D, exposure to sunlight, renal production of 1,25-$(OH)_2D_3$, intestinal absorption of calcium, and the ability to adapt to a low calcium diet may all be impaired in the elderly (Tsai et al, 1984; Reid et al, 1986; Bouillon et al, 1987). Ebeling et al (1992) have also shown that a decrease in the concentration of vitamin D receptors (VDRs) in intestinal mucosa with age in women may also contribute to impaired intestinal responsiveness to endogenous 1,25-$(OH)_2D_3$. As a consequence, secondary hyperparathyroidism often occurs with ageing and can contribute significantly to bone loss. Vitamin D levels have been shown to vary both between and within populations (McKenna 1992; van der Wielen et al, 1995), exhibiting a marked seasonality with lower levels seen in the winter months. These findings could account for some of the geographic and seasonal variation seen in osteoporotic fracture rates.

I.3.3.3 Smoking

Smoking has been reported to be a risk factor for osteoporotic fractures at the hip, spine and wrist (Daniell 1976; Williams et al, 1982; Seeman et al, 1983). In the Study of Osteoporotic Fractures (SOF), current smokers have been shown to have a 2-fold increased risk of hip fracture compared to non-smokers or former smokers (Cummings et al, 1995). A recent meta analysis of 29 cross sectional studies has also confirmed a cumulative effect of smoking on reductions in BMD and increases in hip fracture risk with age (Law and Hackshaw 1997).

Smoking is, however, associated with other diseases that may impact on hip fracture risk. Adjustment for effects such as poorer health, weight loss, reduced quadriceps muscle strength, and reduced exercise levels diminished significantly the risk of fracture associated with current smoking (Cummings et al, 1995). There is, however, evidence from other studies that smoking does have a direct influence on BMD independent of these other associations (Law and Hackshaw, 1997). Slemenda et al (1989) have shown that in premenopausal women, smoking causes reduction in BMD which is more marked at skeletal sites with a high trabecular bone content such as the lumbar spine and distal radius. Hopper and Seeman (1994) studied 41 female twin pairs (21 identical, 20 non-identical) discordant for smoking status. The smoking twin had lower BMD than the non-smoking twin in each pair, even after adjustment for potential confounders such as weight, age at menopause, caffeine intake, dietary calcium intake and exercise levels. This effect was most marked at the lumbar spine, with a mean reduction of 9.3%, compared to a reduction of 5.8% from the femoral neck. A dose response effect was also observed at the lumbar spine, with a decrease in BMD of 2%

for every 10-pack-year habit of smoking. Smoking was associated with increases in serum follicle stimulating hormone (FSH) and leutinising hormone (LH), and reductions in serum PTH.

The mechanism by which smoking reduces BMD is uncertain and several theories have been proposed. Smoking is associated with alterations in reproductive function and a slightly earlier menopause of one to two years, and this may contribute to the reductions in BMD (Baron et al, 1990). Smokers also appear to experience more rapid bone loss in the early postmenopausal years (Krall and Dawson-Hughes 1991). Hirota et al (1993) have shown that smokers have a four-fold increased risk of osteonecrosis of the femoral head, suggesting that smoking may cause vasoconstriction and reduced blood supply to the femoral neck. Smoking may also have a direct toxic effect on bone cells. Lastly, elderly smokers have been shown to have an increased risk of falling (Nelson et al, 1994), and this may explain why the risk of hip fracture associated with smoking is greater than that predicted by the reductions observed in BMD.

I.3.3.4 Other factors

Many other environmental factors have been found to be associated with low BMD in epidemiological studies, although results are not always consistent between studies. Excess alcohol is associated with reduced BMD and accelerated bone loss (Slemenda et al, 1992), although mild-moderate intake has been shown to have beneficial effects on bone mass at both spine and hip (Holbrook and Brett-Connor, 1993). Current caffeine intake has been associated with increased risk of hip fracture (Cummings et al,

1995), with evidence of an interaction between age and dietary intake of both calcium and caffeine (Massey 1991).

I.3.4 Risk factors - Chronic diseases

There are collections of heterogeneous conditions that can be associated with increased bone loss, low bone mass and osteoporotic fracture (Table I.6). In some of these diseases the causal relationship with osteoporosis is established such as with thyrotoxicosis, Cushing's disease, severe hyperparathyroidism and hypogonadism. In other conditions, the link with osteoporosis may be mediated through several disease- and therapy-related mechanisms such as in rheumatoid arthritis, gastrointestinal disease, organ transplantation and prolonged immobilisation. Finally, in some conditions the causal relationship and aetiology remain unknown.

I.3.5 Risk factors - Physical characteristics of bone

When studying individuals with and without fractures there is still considerable misclassification and overlap of low and normal bone density results, with an average of only 0.5 standard deviations (SD) between cases and controls. In addition, and more importantly, BMD still fails to explain a considerable portion of the variance in fracture risk (Ott 1993; Marshall et al, 1996). Since the pathophysiology of osteoporosis includes both loss of bone mass and an alteration of trabecular microstructure, it has been suggested that measurement of other facets of bone strength such as the structure, elasticity and composition may improve fracture prediction.

Table I.6 **Recognised causes of secondary osteoporosis**

Endocrine

- Male hypogonadism
- Hyperthyroidism
- Hyperparathyroidism
- Hypercortisolism
- Diabetes mellitus

Amenorrhoea

- Amenorrhic athletes
- Anorexia nervosa
- Hyperprolactinaemia

Drugs

- Steroids
- Anticonvulsants
- Heparin

Neoplastic disease

- Multiple myeloma
- Skeletal metastases

Other conditions

- Transplantation
- Gastric surgery
- Coeliac disease
- Inflammatory bowel disease
- Alcoholism
- Pregnancy associated osteoporosis
- Immobilisation
- Rheumatoid arthritis or other connective tissue disease
- Osteogenesis imperfecta
- Homocystinuria
- Systemic mastocytosis

I.3.5.1 Bone size

Current absorptiometry methods to measure BMD measure the bone mineral content (BMC) within a given area. The measurement is therefore an areal density and not a true volumetric density, and the correction of BMC for area removes some but not all of its dependency on bone size. Bone size may be an important and independent predictor for osteoporotic fracture. Mazess et al (1994) reported that Z-scores for areal BMD of the lumbar spine were significantly better than calculated volumetric Z-scores in discriminating between patients with vertebral fracture and age-matched control subjects. By contrast in SOF, Cummings et al (1994a) showed that areal BMD and estimated volumetric bone density of the hip both had similar predictive values for hip fracture.

I.3.5.2 Hip geometry

Faulkner et al (1993) have also shown that hip geometry is predictive of hip fracture, with an increased risk of 1.7 for each SD increase in the measurement of hip axis length (HAL). This risk was independent of the association between hip geometry and height. Reid et al (1994) have demonstrated in elderly women from New Zealand that the mean HAL and femoral neck length measurements have increased over a 40 year period (1950s to 1990s). This increase was equivalent to approximately 1 SD and could explain the increase in hip fracture rates that have been observed over this time period. The authors speculate that this increase in geometric hip size may be due to improved nutrition before and during puberty, although the possibility of cohort effect cannot be excluded.

I.3.5.3 Bone structure and architecture

Quantitative ultrasound (QUS) is believed to provide a non-invasive measure of bone structure and is based on the theory that when sound waves pass through porous material such as bone they are scattered and absorbed and travel in a manner that depends on the elasticity, stiffness, volume and density of the material (Abendschein and Hyatt, 1970; Antich 1993). Current machines give figures for broadband attenuation (BUA) and velocity of sound (VOS) through bone. In vitro studies have shown that QUS parameters are highly correlated with structural and architectural components such as trabecular volume fraction and thickness, trabecular number, yield strength and load-bearing capacity (Gluer et al, 1994; Bouxsein et al, 1995). A large number of in vivo studies in humans assessing the relationship between QUS and bone mass and/or fracture have also now been reported. Correlation coefficients between QUS and BMD have ranged from r = 0.14 to 0.88, with most studies finding correlations in the r = 0.40 to 0.70 range (Gregg et al, 1997). These findings suggest that QUS is not a direct surrogate measure of BMD, and may represent a measure of bone structure. At least 27 cross-sectional studies have demonstrated lower QUS measurements in women with prevalent osteoporotic fractures compared to age-matched controls (Gregg et al, 1997). Odds ratio of spine and hip fractures ranged from 1.4 to 3.7 for each SD decrease of either BUA or VOS (Bauer et al, 1995; Gluer et al, 1996). Longitudinal studies have also provided support that QUS is a measure of future osteoporosis and fracture risk (Porter et al, 1990; Heaney et al, 1995; Huang C et al, 1998). A large French population study in the elderly demonstrated that QUS measurement were also of greater predictive value for hip fracture than BMD (Hans et al, 1996).

I.3.6 Risk factors - Racial and genetic factors

There is a wide variation in the prevalence of osteoporosis and in osteoporotic fracture rates between ethnic and racial groups (Melton 1995b). Variation in environmental factors such as diet, physical activity and exposure to ultraviolet light have been suggested as possible reasons for these differences, although none of these factors seems sufficient to fully explain the observed differences. This suggests that genetic factors may play an important role in determining these racial and ethnic differences.

Osteoporotic fracture rates are increased in those of Northern European descent, with the highest rates being observed in Scandinavia (Elffors et al, 1994). The significantly lower incidence of hip fracture among blacks was first noted by Gyepes et al (1962), with subsequent work confirming that Blacks in the US and Africa have fewer vertebral and hip fractures than whites (Moldawer et al, 1965; Solomon 1979; Farmer et al, 1984; Kellie et al, 1990).

This difference in fracture rates has been assumed to be due in part to Blacks having higher BMD compared to whites, with a mean increase of 8% (range 4-12%) (Cohn et al, 1977). This racial difference has been shown in one study to exist already during childhood (Bell et al, 1991) and at least from puberty onward (Luckey et al, 1989). The mechanism by which BMD is greater in Blacks remains, however, unknown. Various studies have explored differences in hormone concentrations (Meier et al, 1992; Dawson-Hughes et al, 1993; Kleerekoper et al, 1994), calcium metabolism (Clemens et al, 1982; Bell et al, 1985; Meier et al, 1991; Bell et al, 1993), body mass (Ortiz et al, 1992), and body composition (Nelson et al, 1991). As noted previously, current

measures of BMD using dual energy xray absorptiometry (DXA) are two-dimensional areal measurements rather than true "density" values. Although BMD reduces the influence of bone size on BMC by normalising values for areal projection, it does not account for changes in bone thickness and remains therefore confounded by bone geometry. Racial differences in BMD may therefore be attributed to differences in bone and skeletal size. Studies have shown that the racial differences in BMD reduce considerably when statistical corrections were made for body size (Nelson et al, 1991; Tobias et al, 1994; Davis et al, 1994), with up to 50% of the variance in BMD being attributable to differences in body size (Nelson et al, 1991). Simple adjustment for height may not be ideal as Blacks have greater leg length but shorter trunk length than white, whereas Asians have the same trunk length as whites but a shorter leg length. Ideally size adjustment should be done using the dimensions of the structure actually measured although this is not practical with the current methods for bone densitometry. These data suggest the importance of bone size as an important indicator of bone strength, although the factors responsible for the ethnic/racial differences in bone size remain to be determined.

Ethnic differences have also been observed in hip geometry, and this may also contribute to the observed racial differences in hip fracture (Cummings et al, 1994b). Similarly, a lower risk of falling has been reported in Black elderly women compared to white and this may also contribute to the racial differences in hip fracture (Tinetti et al, 1988).

I.4 Genetic epidemiology of osteoporosis

The observed racial differences in BMD and fracture risk support the hypothesis that population specific factors may play an important role in determining risk of osteoporosis. This may be due to both genetic and environmental components acting either directly on BMD or on intermediate phenotypes related to bone mass. Alternatively these factors may be acting on other independent phenotypes which are themselves correlated with the risk osteoporosis and fracture. The relative importance of genetic factors in the determination of osteoporosis and bone mass can be quantified through the use of appropriate twin or family based studies.

I.4.1 Family Studies in Osteoporosis

I.4.1.1 Aggregation of bone mass in normal families

The importance of genetic determinants of osteoporosis comes from observations that the disease tended to segregate within families. Early studies examined correlation coefficients (r) between mother-daughter pairs (Table I.7) for non-invasive measurements of skeletal size and BMD using single photon X-ray absorptiometry (SPA).

Table I.7 **Summary of mother-daughter studies examining axial and peripheral BMD measurements**

	No. mothers	Mean age ± SD (yrs)	No. daughters	Mean age ± SD (yrs)	BMD measurement
Lutz (1986)	26	55 ± 4	26	26 ± 5	SPA forearm
Sowers (1986)	34	56 ± 10	36	31 ± 9	SPA forearm
Tylavasky (1989)	84	44.2 ± 0.4 *	84	18.6 ± 0.1 *	SPA forearm
Lutz (1990)	37	52 ± 7	37	25 ± 4	DPA spine and hip
Krall (1993)	40	60 ± 6	40	31 ± 6	DXA spine, hip and total body SPA forearm SPA calcaneus
Kahn (1994)	27 27	49.7 ± 3.6 76.9 ± 6.7	27 27	23.9 ± 4.1 49.7 ± 3.6	SPA forearm
McKay (1994)	41 24	38.8 ± 4.4 67.3 ± 6.7	41 24	11.8 ± 2.1 40.0 ± 5.4	DXA spine and hip
Jouanny (1995)	129	41.9 ± 3.6	98	18.1 ± 2.0	DXA total body
Lonzer (1996)	16	-	28	-	DXA spine and hip
Ferrari (1998)	138	40.0 ± 4.0	138	8.1 ± 0.7	DXA spine and hip
Danielson (1999)	207	71 .7 ± 4.6	207	48.5 ± 7.3	DXA spine, hip and calcaneus

* Standard error (SE)

Lutz et al (1986) demonstrated that radial bone mass was significantly correlated between mother and daughter pairs, with heritability (h^2) estimates of 0.57 for measurements of bone mass/width and 0.72 for bone mass. In a study of similar size and design, however, Sowers et al (1986) observed no familial correlation in forearm bone mass. Tylavsky et al (1989) reported significant correlations in radial bone mass at both mid ($r = 0.34$) and distal ($r = 0.32$) sites, that remained significant after adjusting for age and body mass index (BMI). Kahn et al (1994) studied 27 triads of mother, daughter and grandmother for possible genetic influence on distal and proximal forearm BMD. At the proximal site a significant correlation was found between the mother-grandmother pairs ($r = 0.47$), and although the mother-daughter correlation was 0.33 this failed to reach statistical significance due to the small numbers in the study. Significant correlations between the three generations were found, however, for measures of skeletal size, obesity and muscle strength in the hand.

Lutz and Tesar (1990) first observed familial correlations in BMD at the spine and hip in a study of 37 mother-daughter pairs using dual photon absorptiomerty (DPA). Correlations coefficients ranged from 0.38 to 0.59, and were higher for premenopausal mothers and their daughters compared to the postmenopausal mothers and their daughters. Krall and Dawson-Hughes (1993) studied BMD measurements at several skeletal sites in 40 mother-daughter pairs. Significant correlations were seen at all sites, ranging from $r = 0.30$ for lumbar spine BMD to $r = 0.54$ for total body BMD. A second 3 generation study also found significant correlations for BMD values at the spine and hip between 24 grandmother-mother pairs and 41 mother-daughter pairs (McKay et al, 1994). No significant correlations were observed however for BMD values between the 18 grandmother-daughter pairs even after adjustment for age. A lower mother-daughter

correlation in total body BMD of r = 0.24 was observed by Joanny et al (1995), and probably reflects the fact that values were adjusted for body mass and other environmental confounders. Ferrari et al (1998) examined both spine and hip BMD measurements in prepubertal daughters and their menopausal mothers. BMD was significantly correlated between the mother-daughter pairs (r = 0.22 to 0.36), demonstrating that 18-37% of BMD was directly determined by maternal descent. In the study by Danielson et al (1999), mothers were recruited from the SOF study and results were stratified according to the disease status of the mother: incident peripheral fracture or prevalent vertebral fracture, low BMD but no fracture (i.e. femoral neck BMD T-score < -2.5), or normal BMD (i.e. femoral neck BMD T-score > -1.6). In total, 78 mother-daughter pairs were classified as "normal" and the correlations in BMD measurements for these pairs ranged from r = 0.16 at the femoral neck to r = 0.28. Heritability estimates for this group were not presented, although pooled estimates independent of maternal disease status are given for premenopausal (h^2 range: 50-63%) and postmenopausal daughters (h^2 range: 34 - 48%).

The genetic similarity of mother-daughter and sister-sister pairs is identical, with each subject from a pair sharing on average 50% of their genes with the other family member. The correlation in BMD values within sibs would therefore be expected to be similar for that observed for mother-daughter pairs. Indeed, the closer matching of age and menopausal status within female siblings compared to mother-daughter pairs would be expected to give a "truer" representation of the genetic component acting on BMD.

A small early study in 29 female sibling pairs (mean age 60 ± 14 yrs) identified a significant correlation in crude forearm BMD (r = 0.48), although after adjustment

and/or stratification for age and menopausal status this was no longer significant (Sowers et al, 1986). Further work from this group has also examined the correlation in hip BMD measurements between premenopausal siblings (Sowers et al, 1992). In total data were available from 137 families with the following composition: 2 sisters (n = 96 families), 3 sisters (n = 31), 4 sisters (n = 7), 5 sisters (n = 2), and 7 sisters (n = 1). Variance component analysis was consistent with a polygenic inheritance model for adjusted hip BMD (adjusted for age and BMI), with heritability estimates ranging from 0.67 at the femoral neck to 0.45 at the trochanteric site. No data from lumbar spine measurements was presented.

These studies using data from female first-degree relatives all show a consistent genetic component to BMD. In the absence of data suggesting inheritance of mitochondrial DNA or imprinting (both giving rise to maternal transmission), an autosomal disease transmission is expected. This would imply that the genetic factors acting on BMD would be expected to be contributory from both parents, and that genetic effects would be observed in mother-son in addition to mother-daughter pairs. Comparison of male-female sibling pairs may be affected by the sex differences in BMD acquisition, skeletal size and body fat distribution, and appropriate statistical adjustments need to be utilised. To date, four studies have examined the correlations in BMD between members of nuclear families (Krall and Dawson Hughes, 1993; McKay et al, 1994; Jouanny et al, 1995; Lonzer et al, 1996), and the details of these studies are summarised in Table I.8. Krall and Dawson-Hughes (1993) also present data for 40 daughter-son pairs, although correlations were only significant for os calcis BMD (r = 0.45).

Table I.8 Correlation of BMD measurements across family members

	Mother/ Daughter	Mother/ Son	Father/ Daughter	Father/ Son	Midparent/ Daughter	Midparent/ Son
Krall (1993)						
Total body	0.54 **	0.57 **	0.11	0.24	0.46 #	0.54 ##
Lumbar spine	0.30	0.28	0.16	0.24	0.34	0.37
Femoral neck	0.40	0.47 #	-0.12	0.31	0.22	0.58 **
McKay (1994)						
Lumbar spine	0.34 #	0.25	-	-	-	-
Femoral neck	0.31 #	0.22	-	-	-	-
Jouanny (1995)						
Total body	0.24 #	0.30 ##	0.29 ##	0.18	0.29 **	0.18
Lonzer (1996) *						
Lumbar spine	0.58 #		0.83 ##		0.86 ##	

* Pooling of results for sons and daughters in this stud

# $P < 0.05$ ## $P < 0.01$ ** $P < 0.001$

Overall, these data would support an autosomal genetic contribution to the inheritance of BMD. Differences observed within studies could suggest some sex-specific genetic factors acting on BMD. Differences observed across studies could reflect population-specific genetic components acting on BMD, although they may have arisen because of differences in the sample sizes and ages of the nuclear families, and because of population specific environmental exposures.

I.4.1.2 Segregation analysis of the inheritance of bone mass

Data from family based studies has also allowed segregation analysis to be performed. This allows the inheritance pattern for BMD to be modelled and may subsequently aid the identification of specific loci having effects on the determination of BMD.

Guegen et al (1995) have further analysed the data from the 129 French nuclear families described by Jouanny et al (1995). Segregation analysis rejected the monogenic inheritance model for BMD and was reported to be consistent with a strong polygenic component. The maximum heritability for BMD in this study was 84% which was seen at age 26.4 years, with heritability values of 63.2% (14 years) and 33.8% (53 yrs) being observed at the extremes of the age distribution for the study population. This variability did not appear due to a change in the genetic component with age but was because of an increase in the individual specific variability after the age of 26.4 years, suggesting that the correlation between BMD in two relatives is a function of the ages of each individual in the pair.

In contrast to this study suggesting a polygenic inheritance model, segregation analysis from 302 pedigrees in rural Turkmenia has indicated the possibility of a major gene effect on BMD variance (Livshits et al, 1996). BMD was measured from hand radiographs, with a composite score made for cortical and trabecular bone. The best fitting segregation model suggested the presence of a single gene explaining 50-60% of total variance in BMD, with two co-dominant alleles where the frequency of the allele determining the higher BMD was between 0.30 and 0.38 in this Turkmenian population. Additional evidence that low spine BMD may also be under a dominant, monogenic mode of inheritance comes from data in seven pedigrees with familial osteopenia (total number of subjects = 143, number with spinal osteopenia = 37) reported by Spotila et al (1996). A bimodal distribution to age-adjusted spine BMD values was observed, with simulation data suggesting a disease allele frequency of 0.01.

I.4.1.3 Aggregation of bone mass in families with osteoporosis

The family studies previously described have predominantly all examined BMD correlations within normal subjects. To address the issue that genetic factors may contribute specifically to low bone mass and fracture, several studies have examined these correlations in families with evidence of osteoporosis, and these are summarised in Table I.9. All these studies suggest that there may be site specific genetic factors influencing low bone mass and fracture at the spine and hip. Evans et al (1988) failed to show any reduction in BMD measured at the os calcis using quantitative computerised tomography (QCT) in the first-degree relatives of subjects with vertebral osteoporosis.

although this may merely have been a power issue as this measurement result was only available in 19/35 relatives. Reductions in BMD were found to be greater at the spine compared to femoral neck (7% vs 5%) in daughters of women with vertebral osteoporosis (Seeman et al, 1989), and similarly the reductions in BMD were also greater at the femoral neck compared to lumbar spine in daughters of women with hip fracture (Seeman et al, 1994). In the study by Cohen-Solal et al (1998) all probands were male with a predominance of vertebral fractures. Although the greatest reduction in BMD in first-degree relatives was observed at the spine a significant decrease was also seen at the hip. Danielson et al (1999) studied 72 mother-daughter pairs where the mother had either a prevalent vertebral fracture or an incident fracture of the hip, radius/ulna or humerus. It is not possible to determine the number of mothers that fell into each of these categories, although overall daughters of the women with any fracture had significant reductions in BMD across all measured sites. The mother-daughter correlations in BMD within this fracture group were also higher than had been observed in the women with "normal" BMD, ranging from r = 0.30 at the femoral neck to r = 0.48 at the calcaneus. The lumbar spine correlation was only r = 0.28, although maternal spinal BMD values could have been affected by the presence of prevalent vertebral deformity.

In the absence of clinical or radiographical evidence of osteoporosis and/or fracture, two cross-sectional studies have utilised reported family history of disease to examine whether BMD was reduced in subjects with a positive family history of osteoporosis (Tylavsky et al, 1989; Soroko et al, 1994). These results are summarised in Table I.10.

Table I.9 **Family studies of BMD measurements in relatives of patients with osteoporotic fracture**

	Study				
	Evans (1988)	Seeman (1989)	Seeman (1994)	Cohen-Solal (1998)	Danielson (1999)
No. probands	25	25	74	38	72
Sex ratio (F:M)	19:6	25:0	74:0	0:38	72:0
Fracture status	Vertebral	Vertebral	Hip	Vertebral (n=33) Appendicular (n=5)	Vertebral Appendicular
Mean age ± SD of probands	60 ± 11	67.9 ± 1.5 #	72.9 ± 1.1 #	50.3 ± 11.4	70.8 ± 4.1
No. relatives	35	32	41	73	72
Sex ratio (F:M)	24:1	32:0	41:0	41:32	72:0
Relationship to proband	Siblings Offspring	Daughters	Daughters	Siblings (S) Offspring (O)	Daughters
Mean age ± SD of relatives	43 ± 10	36.9 ± 1.4 #	44.2 ± 2.0 #	43.5 ± 12.4 (S) 27.3 ± 5.4 (O)	47.1 ± 8.0
BMD measurements	QCT spine QCT calcaneus	DXA spine DXA hip	DXA spine DXA hip	DXA spine DXA hip	DXA spine DXA hip SXA calcaneus
BMD reduction in relatives *	1.7 SD - spine	0.7 SD - spine 0.3 SD - hip	0.4 SD - hip	1.3 SD - spine 1.0 SD - hip	0.5 - 0.75 SD

± SE * Compared to age-matched controls

Table I.10 Cross-sectional studies examining BMD in women with reported family history of osteoporosis

	Study	
	Tylavsky (1989)	Soroko (1994)
No. subjects (N)	84	877
Mean age ± SD (yrs)	18.6	74.4 ± 7.4
Definition of family history	**Relationship** • Maternal **History** • Osteoporosis **Fracture site** • Hip, Spine, Colles	**Relationship** • Parental, Sister **History** • Osteoporosis **Fracture** • Any bone age > 50
No. with positive history	20	454
BMD measurement	Radius	Spine/Hip
Reduction in BMD associated with positive family history	Distal: ↓ 2.0 % (NS) Mid: ↓1.7 % (NS)	Spine: ↓ 3.0 % (P = 0.02) Hip: ↓ 1.9% (P = 0.07)

The relationship between family history of osteoporosis and/or fracture and reduced BMD was greatest at the spine and appeared specifically associated with paternal history of disease (Soroko et al, 1994). Potential limitations to these studies include the fact that family history was self-reported and was not able to be validated. Because of the subjects' age, spine BMD measurements may have been artefactually elevated due to the presence of aortic calcification and degenerative spinal disease. The negative findings of Tylavsky et al (1989) also reflect the small sample size and modest power of the study as the magnitude of reduction in BMD seen with a positive family history was similar to that observed by Soroko et al (1994).

I.4.1.4 Family studies of osteoporotic fracture

Studies examining the genetic effect on fracture are limited due to the age of disease onset and the low annual incidence of fracture-estimated at 1-2 women per year sustaining a vertebral fracture for every 100 women aged 65-70 years with low BMD (T score < -2.5) (Seeman & Hopper, 1997). Most data have therefore been derived from reported family history of fracture in subjects who have already experienced an osteoporotic fracture. To date, four cross-sectional and three prospective studies have examined the relationship between self-reported family history of osteoporosis and/or fracture and individual risk of fracture (Tables I.11 and I.12). All these studies are limited by the fact that fracture definition in the relatives was self-reported, whilst in the cross-sectional studies differential recall of family history between fracture cases and controls may also have biased results.

Table I.11 **Cross-sectional studies examining the risk of fracture in women reporting a family history (FH) of osteoporotic fracture in a first-degree relative**

Study	No. subjects (N)	Mean age ± SD (yrs)	Subjects with +ve FH (N)	FH definition	Index fracture	Odds ratio for fracture risk (95% CI) with +ve FH
Gardsell (1989)	48	64 ± 8	All	Maternal vertebral fracture	Vertebral	NS
Soroko (1994)	877	74.4 ± 7.4	454	Parental/sister history diagnosis of osteoporosis and/or fracture aged > 50 yrs	Atraumatic Spine, hip, or wrist Age > 50 yrs	1.28 (0.93, 1.76)
Malmin (1994)	302	62.8 ± 10.1	68	Parental history of hip or wrist fracture	Wrist fracture	1.46 (1.01, 2.11)
Diaz (1997)	6817	61.7 ± 6.9	596	Parental history of hip fracture aged > 50 yrs	Vertebral deformity	1.0 (0.8, 1.3)

Table I.12 **Prospective studies examining the risk of fracture in women reporting a family history (FH) of osteoporotic fracture in first-degree relatives**

	No. subjects (N)	Age ± SD (yrs)	Mean duration of follow up (yrs)	FH definition	No. with +ve FH (%)	Incident fracture	Risk of fracture (95% CI) associated with +ve FH
Cummings (1995)	9516	72 ± 5	4.1	Maternal hip fracture	10%	Hip	2.0 (1.4,2.9)
Torgerson (1996)	1857	Range 47-51	2	Maternal grandmother hip fracture	4.5%	Any	4.5 (1.9, 10.3)
Fox (1998)	9704	72 ± 5	7.1	Parental & sibling fractures at hip and/or wrist	16.7% hip # 12.7 wrist #	Hip Wrist	

The studies by Soroko et al (1994) and Mallmin et al (1994) demonstrate that any increase in fracture risk associated with a reported family history is modest. They also suggest that it is a paternal history of osteoporosis and/or fracture rather than a maternal history that is specifically associated with this increased risk. The number of males with osteoporosis is small and this could have affected the results. The small study by Gardsell et al (1987) showed, however, no difference in the prevalence of fractures in daughters of women with vertebral fracture when compared to controls with a negative family history.

The three prospective studies have all shown an increased fracture risk in women reporting a positive family history of hip fracture (Cummings et al, 1995; Torgerson et al, 1996) and wrist fracture (Fox et al, 1998). The 2-fold increased fracture risk observed by Cummings et al (1995) appeared independent of a subjects' bone mass and of a positive maternal history for other types of fracture associated with falling. This could suggest a genetic component to hip fracture risk that is acting on factors other than BMD and fall-related mechanisms. The study by Torgerson et al (1996) is limited because index case fractures were ascertained only by questionnaire with no validation process, and because traumatic fractures were included in the analysis. It is also unclear why details on hip fractures in maternal grandmothers are only included. Assuming a genetic component to fracture risk, one would anticipate that the risk would be greater with a parental history compared to that in observed in grandparents as parents and offspring share on average 50% of their genes whereas grandparents and grandchildren share on average only 25% of their genes.

In summary, therefore, family studies have shown familial resemblance in BMD measurements at various skeletal sites and are consistent with a major genetic component acting on bone mass. First-degree relatives of subjects with osteoporotic fracture have reduced BMD compared to age-matched controls in a site-specific manner. Family history of fracture, particularly of the hip, also appears to be associated with an increased fracture risk that again appears site specific and independent of BMD. Family studies are, however, confounded inherently by the age differences between parents and their children, particularly with regard to age-related phenomena such as BMD, and by possible major differences in environmental and life-style factors operational during the development of peak BMD. It is because of these problems that twin studies have recently been utilised to aid in the identification of genetic factors underlying the attainment of BMD and the development of osteoporosis.

I.4.2 Twin Studies

Because twins are matched for age and also commonly for environment, twin studies provide a powerful resource for the quantification of the genetic effects acting on complex traits such as osteoporosis. Identical, or monozygous (MZ), twins share 100 % of their genes, and any phenotypic differences within MZ pairs must be due to environmental differences acting between members of a pair. Non-identical. or dizygous (DZ), twins share on average 50 % of their genes, and differences within these twin pairs must be due to both a combination of environmental and genetic differences between subjects. Comparison of the degree of similarity in phenotypes between both MZ and DZ twins gives an indication of the genetic component acting on the trait (Falconer 1981).

I.4.2.1 Twin studies examining bone mass

The first twin study in osteoporosis was described by Smith et al (1973) where bone mass and bone width were measured in the distal third of the right radial midshaft using SPA. The study's subjects included both young (age range 5-18 years) and adult (age range 44-55 years) twin pairs. The variance in radial bone mass and width within the younger DZ pairs was four times greater than the variance within MZ twin pairs, providing evidence for a significant genetic influence on both traits with heritability estimates of 0.75 (bone mass) and 0.77 (bone width). In the adult twins the within-pair variance in the DZ pairs also exceeded the variance within MZ pairs, although heritability estimates were reduced, 0.49 (bone mass) and 0.45 (bone width). These data suggested

a significant genetic determination of bone mass which appeared stronger in younger subjects. The intra-pair differences in bone mass also appeared to increase with age in both the MZ and DZ twins, although this change was only significant in the case of DZ twins. A major limitation to this study was that the analysis in the younger twins pooled data from both male and female same-sexed twin pairs, as the sample size precluded sex specific analysis. In addition, data were also included on three pairs of Negro twins.

This early work has been confirmed and extended by several workers (Table I.13). In a small study of same sexed twins Moller et al (1978) examined metacarpal morphometry, demonstrating that the mean intra-pair variances of both total and cortical width was 4-5 times higher in DZ than in MZ pairs with an estimated heritability of between 0.7 to 0.8. Again, the small sample size resulted in data for male and female twin pairs being pooled.

Dequeker et al (1987) published the first twin study to examine BMD measurements at both peripheral (distal radius) and axial (lumbar spine) sites. Results were stratified by age (below and above 25 years). A significant genetic effect was demonstrated only for radial BMC in the older twins (8 MZ, 5 DZ pairs), with a heritability estimate of 0.75, whereas spinal BMD exhibited a strong genetic component with heritability estimates of 0.88 only in the younger twin pairs (8 MZ, 9 DZ pairs). Although the authors interpret these results to suggest that genetic and environmental factors may have differing effects on the peripheral and axial skeleton that are age dependent, it is more probable that the study was severely limited by the small number of subjects in each age group. In addition, although all twin pairs are reported to be sex-

matched no details are given on whether results were pooled for males and females and whether female twins were concordant for menopausal status.

The results from other twin studies that have examined BMD at different sites are summarised in Tables I.13 and I.14. The consistent finding of quantitative evidence for genes influencing BMD is unlikely if many genes are involved in the inheritance of the trait (Cavalli-Sforza & Bodmer, 1971). These data would therefore suggest that relatively few genes might be involved in the inheritance of BMD. Bivariate analysis of BMD in premenopausal twins has also been reported to be consistent with one gene or a single set of genes determining bone mass at all the skeletal sites measured (Pocock et al, 1987).

Table I.13 Summary of twin studies

	MZ Twins				DZ Twins				Measurement
	No. pairs	Age range	M:F ratio	Menopausal status	No. pairs	Age range	M:F ratio	Menopausal status	
Smith (1973)	76	5-55	48:28	Pre-pubertal + premenopausal	67	5-55	56:11	Pre-pubertal + premenopausal	SPA radius
Moller (1978)	17	60-75	10:7	Postmenopausal	8	50-75	3:5	Postmenopausal	Metacarpal morphometry
Dequeker (1987)	16	10-65	-	-	14	10-72	-	-	SPA radius DPA spine
Pocock (1987)	38	24-75	6:32	Premenopausal (n=19)	27	24-65	1:26	Premenopausal (n=22)	SPA radius DPA spine/hip
Slemenda (1991)	124	25-80	0:124	-	47	30-74	0:47	-	SPA radius DPA spine/hip
Arden (1996a)	128	50-70	0:128	Postmenopausal	122	50-70	0:124	Postmenopausal	DXA spine/ hip/ radius/total body
Flicker (1996)	37	60-89	0:37	Postmenopausal	32	60-89	0:32	Postmenopausal	DXA spine/ hip/ radius/total body

Table I.14 **Heritability estimates for BMD from twin studies**

	Pocock (1987)	Slemenda (1991)	Flicker (1995)	Arden (1996a)
Lumbar spine				
• rMZ	0.92	0.85	0.74	0.68
• rDZ	0.36	0.33	0.33	0.29
• h^2	0.92	1.05	0.82	0.78
Femoral neck				
• rMZ	0.73	0.81	0.74	0.61
• rDZ	0.33	0.37	0.52	0.19
• h^2	0.73	0.88	0.44	0.84
Ward's triangle				
• rMZ	0.85	0.80	0.73	0.47
• rDZ	0.36	0.38	0.31	0.22
• h^2	0.85	0.84	0.84	0.51
Distal radius				
• rMZ	0.71 *	0.78	0.67 #	0.63
• rDZ	0.50 *	0.43	0.62 #	0.32
• h^2	0.42 *	0.70	0.10 #	0.61
Total body				
• rMZ	-	-	0.76	0.63
• rDZ	-	-	0.37	0.25
• h^2	-	-	0.58	0.76

* Distal radius BMC

\# Total forearm BMD

In summary, cross-sectional twin studies have shown consistently that the correlations for BMD are greater in MZ compared to DZ twin pairs and are consistent with a significant genetic component to the variance in BMD. These studies give, however, little indication as to how genetic factors control the underlying physiological mechanisms that regulate the attainment of BMD.

I.4.2.2 Twin studies examining bone turnover

Mother-daughter studies have previously suggested a genetic influence over serum calcium levels (Lutz 1986), with heritability estimates for ionised, ultrafiltrable and total calcium being 0.93, 0.92 and 0.67 respectively. To date, four studies in two different samples of adult twins have examined for genetic effects on osteoblast function and bone turnover in general.

In a study of pre- and postmenopausal twins (39 MZ pairs, 31 DZ pairs) Kelly et al (1991) observed that up to 80% of the variance in serum OC levels, a marker of bone formation, was attributable to genetic factors. Importantly, in DZ twins, within-pair differences in osteocalcin were significantly related to within-pair differences in BMD at both the lumbar spine and femoral neck. The twin with the higher osteocalcin value was found to have the lower BMD measurement at both skeletal sites. In this study, however, no genetic effect was demonstrated for urinary markers of bone resorption (the hydroxyproline:creatinine and calcium:creatinine ratios).

Further studies in these same twins (with only small additions to numbers) using more recent markers of both formation and resorption have also shown a strong genetic component on bone turnover (Tokita et al, 1994; Harris et al, 1998). Again, within-pair differences in PICP and ICTP in DZ pairs predicted differences in lumbar spine BMD, findings that were independent of their OC levels (Tokita et al, 1994). Multivariate analysis also suggested that the genes specifically influencing BAP only accounted for 16% of the total genetic variance at the lumbar spine and 4% at the femoral neck, suggesting that the potential loci influencing BMD and BAP appear largely independent (Harris et al, 1998). Whether the same were true for genes controlling the other markers of bone turnover was not clear.

Garnero et al (1996a) in a larger study of postmenopausal twins (61 MZ pairs, 59 DZ pairs) with measurements on a wide range of biochemical markers demonstrated more modest effects, finding that genetic contribution was greatest in markers that did not change markedly at the time of the menopause. In addition, in this study only within-pair differences of urinary type I collagen cross-linked N-telopeptide (a marker of bone resorption) was predictive of BMD at spine and hip.

In summary, these data suggest a genetic contribution to cellular processes that are involved in bone homeostasis. Population studies have suggested that biochemical markers of bone turnover may be predictive of subsequent rates of bone loss (Christiansen et al, 1987; Christiansen et al, 1993), although these findings have not been replicated in all studies (Keen et al, 1996). Extrapolation of these positive findings to the twin studies on bone turnover could, however, suggest a genetic contribution to rates of postmenopausal bone loss.

I.4.2.3 Twin studies examining rates of change in bone mass

Only two twin studies have examined rates of change in BMD with conflicting results. In a cohort of 46 elderly (age range 60-71 years) male twins (25 MZ and 21 DZ), Christian et al (1989) found no evidence for a genetic effect on change in forearm bone mass, with two measurements taken over a 16 year period. Within-pair correlations (rMZ/rDZ) for the rates of change in forearm BMD were significant for both zygosities (rMZ = 0.35; rDZ = 0.43), although the correlations were not greater in the MZ compared to DZ twin pairs. These data suggested that factors common within twin pairs such as common environmental influences might be important in determining age-related bone loss in males. Further work from the same group also showed that these correlations were reduced by 25-35% after adjustment for smoking, alcohol use, dietary calcium intake and exercise levels (Slemenda et al, 1992).

Conflicting results have been presented by Kelly et al (1993) in a longitudinal study over 1-5 years in male and female twins (16 MZ and 16 DZ pairs). rMZ values were greater than rDZ for the percentage rates of change in lumbar spine BMD. Similar but weaker effects were also seen at the trochanteric and Ward's triangle sites although not at the femoral neck. The authors suggest that the genetic effect on rates of change in bone mass may therefore be greater at trabecular rather than cortical bone sites. There are, however, several concerns about the findings of this study. The number of subjects in this study is small and the stability of the analytical models is therefore questionable. The twins were predominantly premenopausal and rates of change in BMD were small, ranging from -0.02 %/yr at the spine and -1/13 %/yr at the trochanter.

Given that BMD was measured using DPA with coefficients of variation ranging from 2.6 % to 4.1 % across the skeletal sites, it is debatable whether these rates of change were significant given the short duration of the study.

In summary, to date twin studies have not revealed any clear genetic contribution to age or menopause related bone loss. Larger longitudinal studies in postmenopausal twins will be required to test this hypothesis.

I.4.2.4 Twin studies examining osteoporotic fracture

To date only one study has specifically examined the genetic influence on osteoporotic fractures (Kannus et al, 1999). This study examined data from 2308 MZ and 5241 DZ pairs, with fractures sustained over the age of 50 years being derived from hospital coding. Despite the large sample size, only 786 fracture subjects were identified, representing only 5.2% of the total. With a 25-year prospective study this figure appears low and suggests incomplete ascertainment. Because of this small number, fractures across all sites were pooled, and it was not possible to undertake site-specific analysis. In addition, spinal fractures would have been clinical and probably symptomatic, as radiological survey was not undertaken in all subjects. Although the results overall were reported not to support a significant genetic contribution to fracture risk, sex-specific analysis did suggest a genetic contribution in male twins. Further studies will be required to address this issue in more detail.

I.5 Candidate gene studies in osteoporosis

As has been illustrated, osteoporosis is a complex disease that has been shown to have a strong genetic component. The specific genetic elements to consider in such a disease model include:

1) The number of genes involved
2) The frequency of the predisposing alleles
3) The respective effects attributable to specific alleles
4) The combined effects of allelic interactions
5) The background or environmental influence on the allele effects.

As osteoporosis is a disease of later life there is little segregation analysis data from disease affected pedigrees to indicate the mode of transmission. Most data have either been acquired from twin studies or through quantitative trait analysis within large, normal pedigrees. Varying segregation models have been proposed (Geugen et al, 1995; Lipshits et al, 1996; Spotila et al, 1996), although it is now believed that several genes with frequent alleles having relatively small effects and interacting with environmental factors are likely to account for most of the genetic component of the disease (Pocock et al, 1987; Slemenda et al, 1991). At present there is no strong evidence to support a single gene effect on bone mass within the general population. Rare monogenic disorders (i.e. osteogenesis imperfecta) associated with a considerable increase in fracture risk in affected individuals are only observed in a tiny proportion of patients with osteoporosis (Spotila et al, 1991; Spotila et al, 1994).

At any given genetic locus many different forms of a gene representing individual mutations may exist, and these are termed alleles. Many genes have one or several alleles represented at high frequencies in populations and such loci are said to be polymorphic. Typically a gene is defined as being polymorphic when its most frequent allele has a population frequency of less than 0.95, although a less stringent criterion of 0.99 is sometimes used (Ott 1992). This allows for the wide variation in the phenotypic expression of genetic traits that is observed: some alleles being associated directly with disease processes, while others contribute to the "normal" genetic variation seen in all populations. At the deoxyribonucelic acid (DNA) level, the most common polymorphisms are generally based on single nucleotide changes, resulting in a biallelic system. Two other types of polymorphism also occur within the human genome. Variable number tandem repeats (VNTRs) occur because of variable numbers of short oligonucleotide sequences. These VNTRs are much more polymorphic than single nucleotide substitutions (Nakamura et al, 1987), although they occur less frequently throughout the genome. Microsatellites consist of base pair (bp) repeats of short sequences (up to 6 bp) and exhibit wide variability (Weber and May, 1989). Most of the microsatellite polymorphisms identified to date have been based on dinucleotide repeats $(dC\text{-}dA)_n$.

Genetic association studies will allow the comparison of candidate gene polymorphisms between patients and appropriate controls (Lander and Schork,1994). Candidate genes can be tested for association with major traits such as BMD or fracture, and in addition they can also be tested against intermediate phenotypes such as bone turnover and other local, cellular processes. Knowledge of bone pathophysiology provides insight into the large number of proteins that are actively involved in

maintenance of skeletal integrity and the regulation of bone homeostasis. The genes that encode these proteins are therefore all potential candidate genes for the genetic regulation of BMD, and polymorphisms of these genes may contribute to the population variation of BMD and subsequent risk of osteoporosis.

In this thesis, four candidate genes have been examined for polymorphic association with BMD and risk of osteoporosis. The background to the work on these candidate genes is presented in the next sections.

I.5.1 Vitamin D receptor

Vitamin D_3, or cholecalciferol, is a steroid prohormone made in the skin by ultraviolet irradiation of 7-dehydrocholesterol. Metabolism in the liver and kidney converts cholecalciferol to the active metabolite calcitriol, 1,25-$(OH)_2D_3$. Although 1,25-$(OH)_2D_3$ functions as an active hormone with diverse actions in a variety of tissues and cell types, most work to date has concentrated on its physiological regulation of calcium and phosphate homeostasis by bone, intestine and kidney. This is mediated through the VDR, (DeLuca 1992) although recent studies have indicated that 1,25-$(OH)_2D_3$ may also influence cellular processes through a second, non-genomic pathway that is not dependent on its receptor (Swain et al, 1993).

I.5.1.1 Structural organisation of the vitamin D receptor

The VDR is a member of the steroid, thyroid and retinoic acid receptor superfamily (Mangelsdorf et al, 1995). All these receptors have a conserved structure, with the principal functional domains that effect the receptor's action including those for hormonal ligand binding, heterodimerisation, DNA binding/nuclear localisation, and transcriptional activation (Figure I.3). Comparison of VDR protein sequence across species reveals significant similarity. The greatest degree of conservation occurs in those domains of known functional significance.

The human VDR (hVDR) possess a conserved DNA binding domain consisting of two zinc finger motifs (residues 24-90). This structural motif is similar to the helix-turn-helix DNA binding motif found in a number of other proteins that carry out related regulation of transcription. Within the intervening sequence of the two zinc fingers is a cluster of five basic amino acids (residues 49-55). This finding is unique to the VDR class of steroid hormone receptors. This cluster of amino acids is believed to play important roles in the regulation of DNA binding (Rastinejad et al, 1995) and nuclear localisation (Hsieh et al, 1998). In addition, this segment includes ser-51, a site of hVDR phosphorylation by protein kinase C that could result in modulation of both DNA-binding and nuclear localisation of VDR *in vivo* (Hsieh et al, 1991; Darwish et al, 1993).

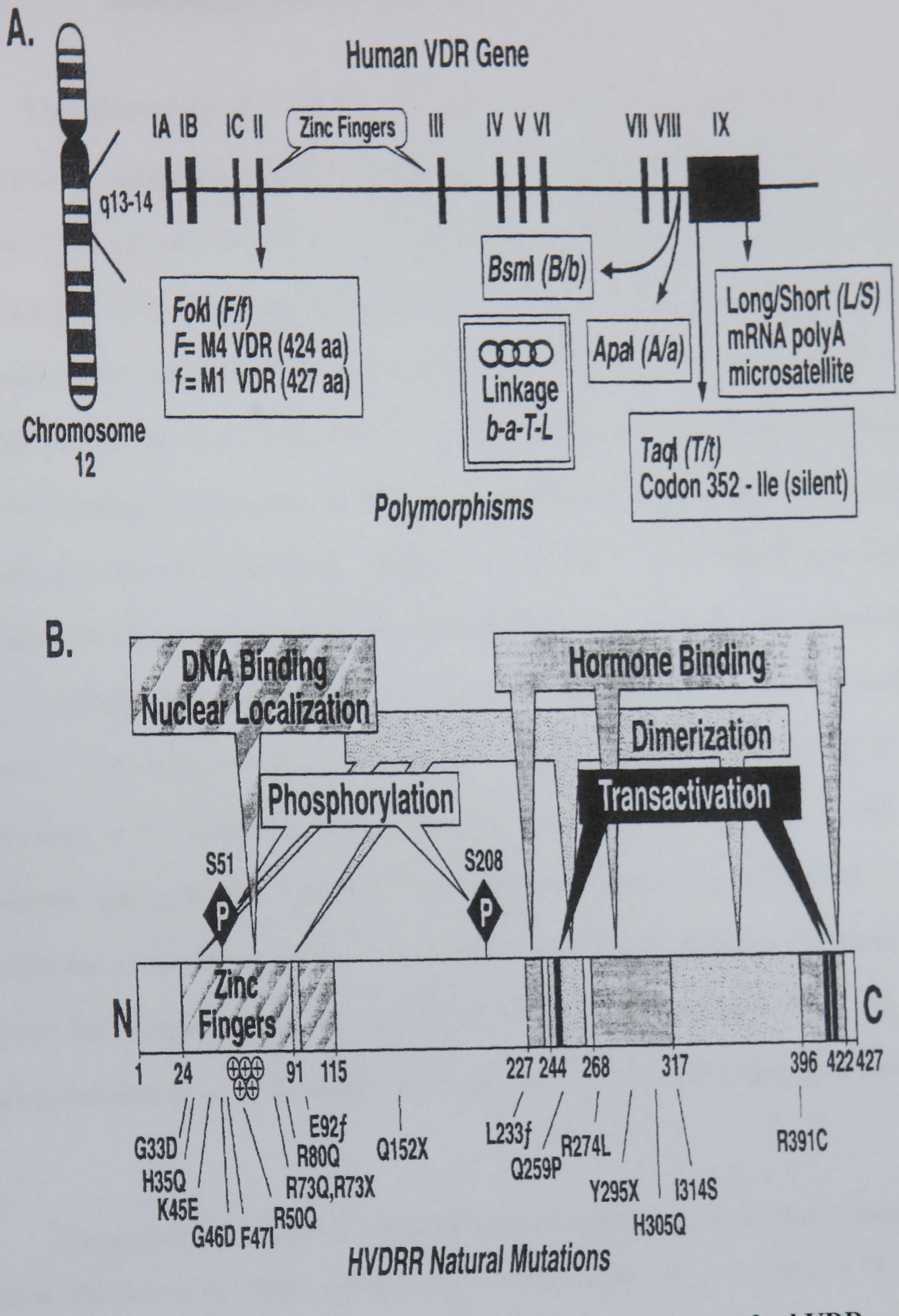


Figure I.3 **Schematic view of genomic and amino acid sequences for hVDR**

(A) hVDR gene demonstrating 11 exons and known polymorphisms

(B) Amino acid sequence with known natural point mutations in human patients

(taken from Haussler et al, 1998)

I.5.1.2 Molecular function of the vitamin D receptor

The classical mechanism of action for 1,25-$(OH)_2D_3$ involves binding of the vitamin to its intracellular receptor, localisation of the ligand-receptor complex to the nucleus of the cell and interaction with a specific regulatory nucleotide sequence within chromosomal DNA. This complex association then induces the transcription of specific messenger RNA (mRNA) coding for proteins which carry out the cellular functions of vitamin D (Darwish and DeLuca 1993). Phosphorylation of specific serine residues in the DNA binding domain of the VDR appears to also be required prior to activation of transcription (Darwish et al, 1993). Carlberg et al (1993a, 1993b) identified two classes of vitamin D response element that are activated either by VDR binding as a homodimer (i.e. VDR/VDR) or as a heterodimer (i.e. VDR with retinoid X receptor or other nuclear factors). The motif GGGTGA arranged as a direct repeat with a spacing of six nucleotides, or as a palindrome without spacing, or as an inverted palindrome with a 6 nucleotide spacing confers induction of transcription mediated by VDR alone. The second class of element activated by the heterodimer complex comprises a 6 nucleotide half-site direct repeat (GGTCCA, AGGTGA or GGGTGA) separated by 3 bp, this spacing contributing to the specificity of the response element to its respective receptor.

The actions of vitamin D on skeletal bone remodelling have been extensively studied (DeLuca et al, 1992). Vitamin D stimulates osteoblast production of OC, and serum levels of this protein are used as an indicator of osteoblast function and of bone formation. The cloning of the human OC gene allowed the identification of a vitamin D response element approximately 500 bp upstream of the promoter (Morrison et al. 1989).

I.5.1.3 Chromosomal location and structural organisation of the hVDR gene

The hVDR was initially cloned by Baker et al (1988), and has been localised to chromosome 12q12-14 by somatic cell hybridisation (Labuda et al, 1992; Szpirer et al. 1991). It is interesting to note that the gene for psuedodeficiency vitamin D rickets (PDDR) due to a deficiency in 25-hydroxycholecalciferol-1-hydroylase maps to the same region. Labuda et al (1991) used a biallelic polymorphism and a VDR cloned DNA (cDNA) probe to demonstrate linkage between the VDR locus and the PDDR locus (lod score 1.94). In addition, no recombination was observed between the VDR locus and the type 2 collagen α1 (COL2A1) gene (lod score 1.94).

Miyamoto et al (1997) characterised the hVDR gene and its promoter by isolating lamda-phage and cosmid clones spanning more than 100 kilobases (kb). The chromosomal hVDR gene encompasses approximately 75 (kb) of genomic DNA and contains 11 exons (Figure I.3). The non-coding 5' end of the VDR gene includes exons 1A, 1B and 1C, while its translated product is encoded by the additional 8 exons (2-9). Three unique mRNA isoforms are produced as a result of the differential splicing of exons 1B and 1C. The DNA sequence upstream of exon 1A is GC-rich and does not appear to contain a TATA box, although several binding sites for the transcription factor SP1 and other activators have been noted. The bulk of the leader sequence of 115 nucleotides is contained in the first exon (Baker et al, 1988). Exon 2 contains the remainder of the leader sequence, the translation initiation codon and the first of the two zinc finger structures. A polymorphic sequence within exon 2 also determines the presence or absence of an alternative translation start site, resulting in a hVDR protein

truncated by 3 amino acids. Exon 3 contains the second finger. thus completing the DNA binding domain of the VDR. Exons 4-6 comprise the nucleotide sequence for the "hinge" portion of the VDR molecule and are located in a cluster. A unique feature of the hVDR gene is the presence of an additional exon (5) that is not found in the nuclear receptor genes and encodes residues 155-194. This region of the VDR protein is more expansive than the corresponding segment in the other nuclear receptors, suggesting that the VDR may have acquired a novel exon of unknown function as it diverged from the other nuclear receptors during evolution (Miyamoto et al, 1997). The final three exons are also located in a cluster and encode the ligand-binding domain. Exon 9 is the largest of the VDR exons and contains the final 250 nucleotides of coding sequence as well as 3.2 kb of 3' non-coding sequence (Baker et al, 1988). Two polyadenylation signals AATAAA are present 25 bp and 70 bp upstream of the poly-A tail respectively.

I.5.1.4 Hereditary disorders associated with mutations in the VDR gene

In total, 18 point mutations have been described in hereditary vitamin D resistant rickets patients (Figure I.3). Single base-pair mutations within exons coding for the DNA binding domain result in disrupted action of 1,25-$(OH)_2D_3$, and present phenotypically as hereditary vitamin D resistant rickets (Yagi et al, 1993). A missense mutation in exon 3 causing an amino acid substitution (Arg to Gln at amino acid position 77) has been reported in two affected siblings with hereditary vitamin D resistant rickets (Malloy et al, 1994). This region codes for the base of the second zinc finger that is critical for DNA binding of the receptor. Missense mutations in the hormone-binding domain of the VDR gene have also been described. A substitution of Leu to Arg at

amino acid position 271 results in a receptor which has reduced binding affinity, with expression analysis demonstrating that gene transcription could be elicited by the mutant VDR only in the presence of a 1,000-fold higher concentration of 1,25(OH)2D3 (Kristjansson et al, 1993). Several truncated forms of the VDR have also been recognised in patients with hereditary vitamin D resistant rickets (Wiese et al, 1993) where nucleotide substitutions produce premature stop codons resulting in messenger RNA transcripts that lack the full hormone binding domain.

I.5.1.5 VDR null mice

VDR knockout mice have recently been created by two groups (Yoshizawa et al, 1997; Li et al, 1997). Heterozygotes appeared normal, whereas homozygous VDR -/- mice displayed a phenotype similar to HVDRR. Homozygous mice were born normal, subsequently developing symptoms and signs of rickets/osteomalacia and secondary hyperparathyroidism after weaning. These included low bone mass, hypocalcaemia, hypophosphataemia, elevated PTH, and a 10-fold elevation in 1,25-$(OH)_2D_3$ coincident with extremely low 24,25$(OH)_2D_3$. Affected homozygotes either died within 15 weeks or exhibited near normal survival rates for up to 6 months, differences that may have been attributable to variations in either diet or environment. Partial phenotypic rescue of the VDR knockout mice was possible with dietary manipulation of the blood calcium and phosphate levels. PTH was normalised and bone mineralisation was greatly improved with these measures, although alopecia and skin changes such as dermal cysts persisted.

I.5.1.6 Restriction fragment length polymorphisms in the VDR gene

The cloning of the human cDNA allowed identification of restriction fragment length polymorphisms (RFLPs) within the gene. Faraco et al (1989) identified an *ApaI* RFLP at the human VDR gene locus with bands at either 7.9 kb or 3.0 kb. Co-dominant inheritance of this dimorphism was demonstrated in one family of 7 individuals Two further RFLPs were detected by Morrison et al (1992) using the restriction endonucleases *BsmI* and *EcoRV* and a 2.1 kb VDR cDNA probe. The RFLPs were coded as "Aa" (*ApaI*), "Bb" (*BsmI*), and "Ee" (*EcoRV*), where the upper case letter signifies absence of the restriction site and the lower case letter signifies presence of the site. The genotypes of 182 unrelated individuals were assessed using all three VDR RFLPs and this demonstrated a strong degree of co-association indicating linkage disequilibrium (LD) at this locus. The RFLPs were highly associated such that genotype "AA" was found with "BB" and "EE" at frequencies of 83% and 92% respectively. Correspondingly, genotype "aa" was found with "bb" and "ee" at frequencies of 61% and 72% respectively. Hustmyer et al (1993) detected an additional VDR RFLP using the restriction endonuclease *TaqI* and a 1.4 kb full length cDNA of the VDR open reading frame. For the *TaqI* enodonuclease, two variant bands were detected at 2.0 kb and 2.2 bb. Mendelian inheritance was demonstrated in one family of 8 individuals. In this study Hustmyer et al (1993) found that the RFLPs differed between American white Caucasians, Blacks and Asians (Table I.15).

The most informative RFLPs (*BsmI*, *ApaI* and *TaqI*) were subsequently mapped to the 3' region of the gene using Sau-3A restriction sub-fragments of the VDR cDNA (Morrison et al, 1994). The *BsmI* and *ApaI* sites lie within intron 8 and do not alter the VDR messenger RNA sequence, whilst the *TaqI* site is within exon 9 (Figure I.3). It is, however, a synonymous codon change (i.e. ATT to ATC) and does not alter the amino acid sequence (isoleucine) at this position. There was, on average, 98% concordance for

the haplotypes BAt and baT (Morrison et al, 1994). A microsatellite poly(A) repeat has also been mapped to the 3'-untranslated region (UTR), approximately 1 kb upstream of the poly(A) tail (Morrison et al, 1994). Multiple (≥ 12) allelic variants of this microsatellite have been detected and classified into two groupings, long (L) and short (S), based upon the length of the repeat (Ingles et al, 1997a; 1997b). The L grouping appears significantly linked to the *TaqI* T allele.

Near the 5' region of the hVDR, a *FokI* restriction endonuclease site has been identified (Saijo et al, 1991; Gross et, 1996). The presence of the restriction site dictates the expression of the 427-residue, M1 isoform of the VDR (so named because it contains an ATG methionine translational start site corresponding to codon #1). An evolutionary more recent polymorphism has been reported in which the ATG codon #1 and the *FokI* restriction site are changed, causing a 424-residue isoform of the receptor to be translated (Gross et al, 1998).

Table I.15 Summary of frequencies of polymorphisms at the VDR locus in different racial groups

RFLP	Racial group	n	Allele frequency			Heterozygosity	Reference
			A1	A2	A3		
ApaI	American Caucasians	153	0.56	0.44			Faraco et al 1989
	Australian Caucasians	256	0.49	0.51			Morrison et al 1992
	American Caucasians	85	0.50	0.50		0.48	Hustmyer et al 1993
	American Blacks	19	0.45	0.50	0.05	0.63	Hustmyer et al 1993
	American Asians	16	0.22	0.78		0.31	Hustmyer et al 1993
BsmI	Australian Caucasians	182	0.44	0.56			Morrison et al 1992
	American Caucasians	85	0.44	0.56		0.55	Hustmyer et al 1993
	American Blacks	19	0.21	0.79		0.42	Hustmyer et al 1993
	American Asians	16	0.06	0.94		0.12	Hustmyer et al 1993
EcoRV	Australian Caucasians	255	0.49	0.51			Morrison et al 1992
	American Caucasians	85	0.49	0.51		0.46	Hustmyer et al 1993
	American Blacks	19	0.32	0.68		0.63	Hustmyer et al 1993
	American Asians	16	0.22	0.78		0.31	Hustmyer et al 1993
TaqI	UK Caucasians	190	0.40	0.60			Spector et al 1992
	American Caucasians	85	0.39	0.61		0.48	Hustmyer et al 1993
	American Blacks	19	0.21	0.79		0.42	Hustmyer et al 1993
	American Asians	16	0.03	0.97		0.06	Hustmyer et al 1993

N = number of subjects studied A1= absence of restriction site A2 = presence of restriction site

I.5.1.7 hVDR gene polymorphisms and osteoporosis

The OC gene on chromosome 1 has a VDR hormone response element in its promoter region, and 1,25-$(OH)_2D_3$ is known to induce OC protein synthesis (Morrison et al, 1989). Production of OC may be regulated by functionally different alleles of the VDR gene on chromosome 12, given that Kelly et al (1991) had shown high heritability estimates for circulating levels. In an association analysis Morrison et al (1992) demonstrated that VDR RFLPs predicted circulating osteocalcin levels in 182 unrelated subjects, with these results being independent of age or menopausal effects. Morrison et al (1994) subsequently examined the relationship between VDR gene alleles and bone mass at spine and hip in a study of 64 MZ and 49 DZ female twin pairs. VDR genotype was assigned using the polymerase chain reaction (PCR) and restriction endonuclease digestion with the enzymes *BsmI*, *ApaI* and *TaqI*. VDR genotype was specifically associated with BMD at the spine and to a lesser extent at the hip in this group, with the allele "B" (or "t") contributing to low bone mass in an additive or co-dominant model. The magnitude of the effect due to the *BsmI* RFLP was approximately 1 SD of the age-matched population distribution. It was subsequently estimated that the VDR locus might explain up to 75% of the genetic variation in BMD. A similar relationship was seen in 311 randomly selected unrelated women, with the "BB" genotype having about 10-15% (equivalent to 1 SD) lower spine BMD compared to "bb" individuals. The "BB" genotype was over represented in those women with BMD measurements 2 SD below the peak adulthood value (i.e. T score <-2). Extrapolation of the cross-sectional data suggested that women with the low BMD genotype could reach the fracture threshold up to 11 years earlier than the other individuals.

Preliminary functional data from the report by Morrison et al (1994) using minigenes and heterologous promoter and reporter constructs showed increased luciferase activity associated with the haplotype BBAAtt compared to bbaaTT. These differences were suggestive of either increased mRNA synthesis or stability. There were, however, many differences in the sequences used between the constructs (i.e. point mutations, insertions and deletions), and any one of these variations could have affected the in vitro mRNA concentrations.

I.5.2 Oestrogen receptor

Oestrogen action is mediated by oestrogen receptors (ER), ERα and ERβ, which have unique tissue distributions and different affinities for oestrogen agonists and antagonists. The first report of the presence of ERα in bone tissue was made by Eriksen et al (1988) in a study of human cells of osteoblast lineage using Northern blot analysis.

I.5.2.1 Chromosomal location and structural organisation of the ERα gene

Walter et al (1985) cloned and Greene et al (1986) sequenced a cDNA for the entire translated portion of the mRNA for the ER of MCF-7 human breast cancer cells. The cDNA contains 1,785 nucleotides and encodes a protein of 595 amino acids (predicted molecular weight 66,182). The genomic organisation of the ER gene was subsequently described 2 years later (Ponglikitmongkol et al, 1988), consisting of 8 exons spanning 140 kb (Figure I.4).

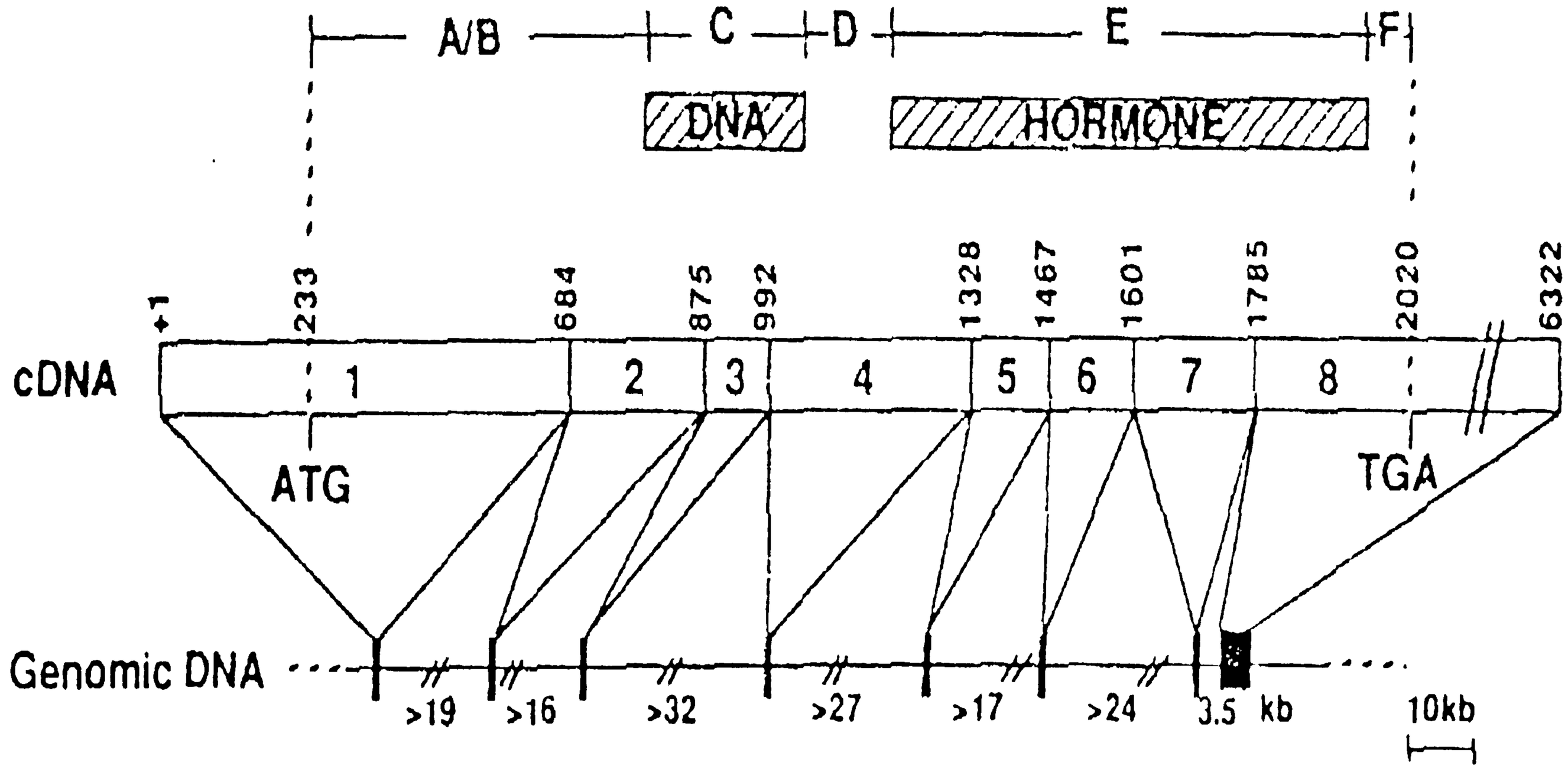


Figure I.4 Structural organisation of the human ER gene

The eight exons are shown as solid boxes (genomic DNA) with the minimum size of each intron indicated between each box. The corresponding portion of the eight exons (1-8) with respect to the ER cDNA is shown above (cDNA) with numbers at the borders referring to nucleotides

The position of the introns appears highly conserved, being remarkably similar to one of the chicken thyroid hormone receptor genes. The human ERα gene was initially mapped to chromosomal region 6q24-27 using in-situ hybridisation (Gosden et al, 1986). Menasce et al (1993) using yeast artificial clones (YAC) and fluorescence in situ hybridisation subsequently provided more accurate localisation to 6q25.1.

A highly conserved 66-amino acid region of the oestrogen and glucocorticoid receptors, which corresponds to part of the receptors' DNA-binding domain (region C), determines the specificity of target gene recognition. This region contains 2 subregions (CI and CII), encoded by two separate exons that are analogous to "zinc fingers". Mader et al (1989) showed, through the study of a chimeric ER, that 3 amino acids located on the C-terminal side of the CI finger played a key role in this specificity.

I.5.2.2 Hereditary disorders associated with mutations in the human ERα gene

The finding of an important functional role for oestrogen and its receptor in the human male skeleton is highlighted in the report by Smith et al (1994) who described a 28 year old male with an oestrogen receptor mutation. The subject presented with marked osteoporosis and biochemical evidence of increased bone turnover. Other phenotypic characteristics included tall stature with incomplete epiphyseal closure, bilateral axillary acanthrosis nigricans and biochemical evidence of insulin resistance with impaired glucose tolerance. Serum sex hormones and pituitary hormones also indicated primary resistance to oestrogen. Molecular analysis subsequently revealed that the patient was homozygous for a C→T mutation at codon 157 in exon 2 which introduced

a premature stop codon (TGA). This was in the A/B domain of the ER protein and would be expected to yield a severely truncated protein with no functional activity.

I.5.2.3 ERα null mice

ER-knockout mice have been generated by disruption of exon 2 of the murine ER gene (Lubahn et al, 1993). Homozygous knockout mice have been shown to have reductions in BMD of 20-25% when compared to wild-type mice (Korach 1994). These findings were seen in both male and female mice, suggesting a role of oestrogen in the development and maintenance of bone mass in the male skeleton.

I.5.2.4 Restriction fragment length polymorphisms in the ERα gene

Gross structural mutations within the human ERα gene appear rare, although a large number of variant and/or atypical receptor transcripts and proteins have been described, particularly in primary breast cancers and established breast cancer cell lines. Variant mRNAs that appear to arise from alternate splicing of the primary ER transcript have been described which lack exon 2, 3, 4, 5 or 7 (Table I.16). Other splice variants include ones containing six additional bp from the 5' of intron 2 (Fuqua et al, 1993). those lacking the 3' nine bp of exon 4, those containing an additional 1 kb of intron 5, and various insertions of approximately 130 bp in exon 5 homologous to the *alu* gene family (Graham et al, 1990). Functional data on these variants suggests that this altered splicing of the ER mRNA may contribute to the clinical behaviour of the tumours and perhaps could serve as a prognostic factor.

Table I.16 Oestrogen receptor RNA splice variants from breast cancer tumours and cell lines

Deleted exon	Source	ER activity	Reference
2	T47D cells	Unknown	Wang et al, 1991
3	T47D cells Human tumours	Dominant-negative Non-functional	Wang et al, 1991 McGuire et al. 1991
4	MCF-7 and ZR75-1 cells	Unknown	Pfeffer et al, 1993
5	ER^-/PR^+ human tumours	Constitutive function	Fuqua et al, 1991
7	T47D cells ER^-/PR^+ human tumours	Non-functional Dominant-negative	Wang et al, 1991 Fuqua et al, 1992

Sequence variation in non-malignant somatic cells has also been examined and several common polymorphisms have been identified. Using RNAse protection assays, Garcia et al (1988) identified a genetic polymorphism within the mRNA region encoding the amino terminal portion of the ER protein. Subsequent direct sequencing identified 2 mutations (Garcia et al, 1989), although the C→T substitution at position 257 was subsequently shown to be artefactual (Taylor et al, 1992). The mutation at position 261 is a synonymous change and does not alter the amino acid sequence. An early small study (Taylor et al, 1992) showed the frequency of the of the ER "B-variant" allele to be 0.04 in Caucasians (n = 22) and 0.07 in Blacks (n = 7). A subsequent study (Schmutzler et al, 1991) showed an identical allele frequency of 0.06 in both women with breast cancer (n = 300) and in those with no history of breast cancer (n = 183). No link between ER genotype and tumour histology and expression of ER could be demonstrated. The B-variant genotype has been associated with an increased rate of spontaneous abortion in women with a history of breast cancer (Lehrer et al, 1990).

although this finding has not been confirmed in case-control study of women with a history of repeated miscarriage (Taylor et al, 1993). A small study in women (n = 88) has also suggested an association between the B-variant allele and hypertension (Lehrer et al, 1993). In this study there were 21 subjects who were heterozygous for the polymorphic allele, giving an inflated frequency of 0.12. This is double that observed in other studies and could suggest selection bias and an unrepresentative study sample.

A *PvuII* polymorphism was identified by Castagnoli et al (1987) using Southern blot analysis. Mendelian inheritance was demonstrated in two Italian families. Hill et al (1989) found no difference in the allele frequencies of the ER *PvuII* polymorphism between DNA samples from human breast tumour samples (n = 188) and blood from healthy controls (n = 53). The allele frequencies were also similar to those observed by Castagnoli et al (1987). Presence of the 1.6 kb allele (allele 1) appeared to be associated with reduced ER expression by breast tumour cells. The 1.6 kb allele was also found to correlate with an older age of diagnosis in 59 breast cancer patients (Parl et al, 1989). In a larger study of 257 breast cancer patients and 145 controls, Yaich et al (1992) failed however to show any relationship between the *PvuII* polymorphism and ER expression on tumour cells or with patient age at diagnosis. The *PvuII* polymorphism has been mapped to intron 1 of the ER gene, lying 0.4 kb upstream of exon 2 (Yaich et al, 1992). The precise functional significance of this polymorphism on ER function and breast cancer is therefore uncertain. Andersen et al (1997) studied leucocyte DNA from 143 patients with familial clustering of cancer (breast and/or ovarian). Three patients were found to have a germline glycine to cysteine substitution at codon 160 of the ER gene. This is believed to represent a polymorphism as this change was also observed in 8/729 controls, split equally between males and females. In the 229 female controls in whom family history of cancer was known, 1 of 2 who had a sister with breast cancer was carrying the variant allele.

A polymorphic dinucleotide repeat $(TA)_n$ has been identified in the promoter region of the ER gene (del Senno et al, 1992). This mapped to position -1174 in relation to exon 1. Co-dominant segregation was observed in 6 families. In total, 17 alleles were identified in an initial analysis of 70 chromosomes from 70 unrelated Italian individuals. The heterozygosity index was 82%.

I.5.3 Type I collagen α1

Type I collagen is the major protein constituent in skin, tendon and bone, and consists of the same protein, containing 2 α1 polypeptide chains and 1 α2 chain. Differences in the collagens from these three tissues are a function of the degree of hydroxylation of proline and lysine residues, aldehyde formation for cross-linking, and glycosylation.

I.5.3.1 Chromosomal location and structural organisation of the COLIA1 gene

Sundar Raj et al (1977) mapped a type I collagen gene to chromosome 17 using cell hybridisation and micro-cell hybridisation. Huerre et al (1982) used a cDNA probe in both man-mouse and man-Chinese hamster somatic cell hybrids to demonstrate co-segregation of the type I collagen 1α (COLIA1) gene with chromosome 17, with subsequent in-situ hybridisation mapping this gene to chromosomal band 17q21.31-q22.05 (Retief et al, 1985). Tromp et al (1988) were the first group to characterise a full-length human COLIA1 cDNA. The COLIA1 gene exhibits structural similarity with

collagens type I α2, type II and III, showing a characteristic structure of a large number of relatively small exons (54 and 108 bp) at evolutionary conserved positions along the length of the triple helical Glycine-X-Y portion (Boedtker et al, 1983). The X and Y stand for the amino acids following and preceding the glycine in the tri-peptide repeat.

I.5.3.2 Hereditary disorders associated with mutations in the COLIA1 gene

Willing et al (1993) observed that cultured dermal fibroblasts from patients with osteogenesis imperfecta (OI) type 1 produced about half the normal amount of type I procollagen. This is because of a decreased COLIA1 mRNA synthesis, indicating a possible defect in this gene in the majority of patients with type 1 OI. In a survey of 40 unrelated probands with OI type 1 where no causative mutation was known, no promoter region mutations were identified with little evidence of sequence diversity (Willing et al; 1995). A large number of mutations and deletions have, however, been described within the coding region of the α1 and α2 type I collagen genes, and Kuivaniemi et al (1997) summarised the sequence data on 317 apparently unrelated OI patients. Most mutations of the type I collagen genes were single bp changes and either changed the codon of a critical amino acid (63%) or led to abnormal mRNA splicing (13%). Most of the amino acid substitutions were those of a bulkier amino acid replacing the obligatory glycine of the repeating Gly-X-Y sequence of the collagen triple helix. Many patients with milder OI showed evidence, however, of a null allele due to a premature stop mutation in the mutant mRNA transcript.

I.5.3.3 COLIA1 gene mutations and osteoporosis

Spotila et al (1991) have identified a single bp mutation (G→A) in the α2 chain of type I collagen, causing an amino acid change at codon 661 of glycine to serine. This mutation was found in a 52 year-old Caucasian woman with low bone mass, thoracic vertebral fracture and 5 previous fragility fractures from the ages of 8 to 35. The patient also exhibited signs that may have been attributable to mild OI (i.e. slight evidence of blue sclerae and mild hearing loss). This mutation was observed in her three sons (age range 24-31 years) who had each sustained one to four fractures as adolescents, although it was not present in DNA samples from the patient's mother (no DNA sample was available from the father) nor in a further 54 unrelated individuals. A subsequent study (Spotila et al, 1994) examined for coding sequence defects in both the collagen type I α1 and α2 genes in 26 subjects (21 female, 5 male). All subjects had low BMD, a positive family history of osteoporosis, and an absence of metabolic bone disease or clinical features to suggest OI. In addition to the sequence defect already observed (Spotila et al, 1991), two unrelated subjects (32 year old female and a 16 year old boy) were found to be heterozygous for a G→C mutation that altered the proline codon at position 27 of the α1 chain to alanine. To study the frequency of this substitution in a larger population, DNA was isolated from 81 normal individuals and a further 37 women with postmenopausal osteoporosis. None of these individuals exhibited this G→C mutation. Four other sequence variants were observed, although these occurred with equal frequency in the osteopenic and normal populations: alanine to proline at position α_2(I)-459, T/C in the asparganine codon at position α_2(I)-3 of the N-telopeptide, A/C in the proline codon at position α_2(I)-392, and T/C at codon α_2 (I)-955 (Constantinou et al, 1990; Strobel et al, 1992).

I.5.3.4 COLIA1 gene polymorphisms and osteoporosis

Mutations within the protein-coding region of type I collagen that cause gross structural changes therefore appear rare in postmenopausal osteoporosis. Sequence changes in regulatory regions of the type I collagen gene may, however, predispose to osteoporosis with alterations in type I collagen synthesis and metabolism. Screening the COLIA1 transcriptional control regions by PCR-SSCP in a sample of 50 subjects, Grant et al (1996) identified 3 polymorphisms in the first intron. Two of these were rare with allele frequencies of approximately 0.04 and 0.03. The third, common polymorphism had an allele frequency of 0.22 and represented a G→T substitution at the first base of a consensus site for the transcription factor Sp1 in the first intron (nucleotide 2046). The functional significance of this polymorphism on type I collagen synthesis is as yet uncertain, although preliminary data suggests that the rarer allele is associated with an increase in transcriptional activity (Grant et al, 1998). This polymorphism has recently been associated with reduced spinal bone mass and an increased risk of prevalent vertebral fracture in two populations from Aberdeen and London (Grant et al, 1996).

I.5.4 Interleukin-1 receptor antagonist

Oestrogen deficiency is associated with an increase in local production of various cytokines and growth factors within the bone marrow and bone cells, and these inflammatory factors appear to play an important role in the development of postmenopausal osteoporosis (Ralston, 1994). Interleukin 1 (IL-1) is a powerful stimulant of bone resorption and a well-recognised inhibitor of bone formation (Dinarello, 1991). IL-1 is a powerful stimulant of bone resorption, activating mature osteoclasts indirectly via a primary effect on osteoblasts and inhibiting osteoclast apopotosis. Monocytes of patients with "high turnover" osteoporosis, the histological

hallmark of postmenopausal osteoporosis, secrete increased amounts of IL-1 (Pacifici et al, 1993). Subsequent observations have shown that this postmenopausal increase in IL-1 activity results from an effect of oestrogen on the production of both IL-1 and its receptor antagonist IL-1ra. Animal studies have also revealed that treatment with IL-1ra blocks the ex vivo osteoclast formation induced by ovariectomy (Kitazawa et al. 1994). The IL-1ra protein binds to IL-1 receptors and competes with both IL-1α and IL-1β without detectable IL-1 agonist effects. This results in the biological activity of these two cytokines being neutralised in physiological and pathophysiological immune/inflammatory responses.

IL-1ra levels are elevated in the blood of patients with a variety of immune, infectious and traumatic conditions. Two forms of the protein exist through alternative splicing of 2 different first exons.

I.5.4.1 Chromosomal location and structural organisation of the IL-1RN gene

The human interleukin 1 receptor antagonist gene (IL-1RN) was first cloned by Carter et al (1990), and subsequently mapped to chromosome 2q using somatic rodent/human cell hybrids (Steinkasserer et al, 1992). The gene consists of 4 exons. Using a YAC containing the IL-1RN gene as a probe in fluorescence in situ hybridisation, Patterson et al (1993) more accurately assigned the IL-1RN gene to 2q14.2. The genes for interleukin 1α, interleukin 1β, and the interleukin 1 receptors also map to the chromosomal region 2q, supporting the view that an early gene duplication event resulted in the creation of an interleukin 1 gene family.

I.5.4.2 Polymorphisms of the IL-1RN gene

Early mapping studies of the IL-1RN gene utilised the presence of polymorphic length variation within the second intron (Steinkasserer et al, 1992). Direct sequence analysis by Tarlow et al (1993) demonstrated that this polymorphism was caused by a variable copy number of an 86 bp sequence (Figure I.5). In total, 5 alleles were identified comprising between 2 and 6 repeats. The 4-repeat (allele A1) and 2-repeat (allele A2) alleles appear most common, while the other alleles occur at a combined frequency of less than 5% (Tarlow et al, 1993; Blakemore et al, 1996). The A2 allele has been found to be associated with a more severe clinical outcome in several inflammatory diseases, including systemic lupus erythematosus (Blakemore et al, 1994), Graves disease (Blakemore et al, 1995), ulcerative colitis (Mansfield et al, 1994), lichen sclerosus (Clay et al, 1994) and alopecia areata (Tarlow et al, 1994). Blakemore et al (1996) have also demonstrated an association between the IL-1RN A2 allele and diabetic retinopathy. The functional significance associated with the 86-bp VNTR is uncertain, although the polymorphic sequence may have direct effects as it is reported to contain three potential protein binding sites; an α-interferon silencer A, a β-interferon silencer B and an acute phase response element (Tarlow et al, 1993).

Additional single nucleotide polymorphisms have also been identified throughout the IL-1RN gene (Figure I.5).

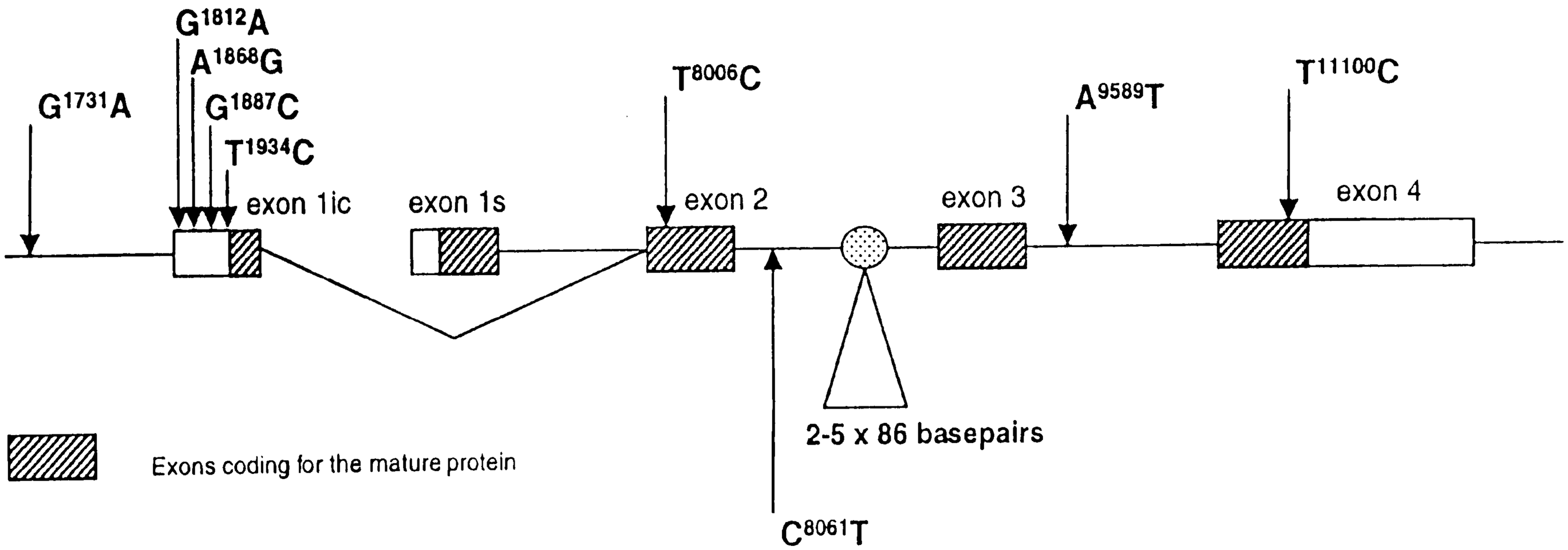


Figure I.5 **Structural organisation of human IL-1RN gene and identified polymorphisms**

I.5.5 Conclusions

At the onset of this thesis limited results on the association between candidate genes and osteoporosis were available. Although preliminary evidence was available for the VDR and COLIA1 genes, these findings were limited to reports from a single study in each case. Cross-sectional association studies also only allow the candidate gene to be tested for association with baseline BMD, but provide little insight into pathophysiological processes. Assessment of longitudinal data may determine, however, whether the candidate gene is specifically associated with bone turnover, rates of bone loss or fracture incidence, thereby providing a possible functional mechanism.

In the absence of a major gene effect on BMD, it is important to simultaneously examine several candidate genes in the same population. This allows assessment of evidence for interactions between genes acting on BMD and contributing to fracture risk.

I.6 Thesis aims

The main aims of this thesis are outlined below.

1. To examine the candidacy of VDR gene polymorphisms on osteoporosis risk

a) To attempt to replicate the original twin studies findings results from Morrison et al (1994) in a different population of Caucasian, postmenopausal twins

b) To extend the association between VDR genotype and BMD to unrelated women from the general population

c) To examine for association between VDR genotype and longitudinal rates of bone loss, and osteoporotic fracture

2. To examine the candidacy of other genes on osteoporosis risk

a) To study the *PvuII* and *XbaI* ER gene polymorphisms for association with BMD and bone loss in unrelated women

b) To examine the COLIA1 gene polymorphism for association with BMD and fracture in a case-control study, thereby attempting to replicate the findings of Grant et al (1997)

c) To assess the IL-1RN VNTR genotype for association with and BMD and bone loss in unrelated women

3. **To examine for evidence of locus interactions on BMD and other intermediate disease-related traits**

a) Analysis of genotype data (VDR, COLIA1, ER, IL-1RN) against osteoporotic phenotypes in both twins and unrelated women

4. **To examine for a genetic association between osteoporosis and osteoarthritis**

a) Analysis of VDR genotype against risk of osteoarthritis at spine and knee in unrelated women

CHAPTER II

METHODS OF INVESTIGATION

II.1 Summary

This chapter presents details of the subjects who have been studied in this thesis and also of the methods utilised in the molecular and statistical analysis. Data on female twins and from a population-based cohort of unrelated women were available at St. Thomas' Hospital for this study. Information regarding the selection and recruitment of these subjects, and of how they compare to women from the UK general population is given. All women completed a detailed osteoporosis risk factor questionnaire and the information obtained via this route is discussed. Full details are also given for the equipment used in the measurement of BMD in all subjects.

In total, four candidate genes were analysed (VDR, ER, IL-1RN and COLIA1). Details of the polymorphisms studied and the molecular biological detection methods used are presented.

Heritability analysis was conducted in the MZ and DZ twins using standardised techniques. A modified sib-pair linkage analysis within the DZ pairs was used to examine for evidence of linkage between the candidate locus and the trait of interest (i.e. BMD). Candidate genes were tested for association with disease-related traits within the population cohorts using standard statistical methods. The underlying models and statistical assumptions for these tests are discussed in detail.

II.2 Subjects

II.2.1 Twin study

Female twins were studied from the St. Thomas' Adult Twin Register. This study was established in 1992 by Dr. Tim Spector to initially study the genetic effects on complex rheumatic diseases such as osteoporosis and osteoarthritis. The study's aims have since expanded to include an understanding of the genetic basis for a wide range of age-related phenotypes across multiple organ systems. Twins were initially obtained from a small registry at the Institute of Psychiatry, London University, although the majority of twins were subsequently recruited directly via national media campaigns. Female twins in the age range 45-70 were invited to attend and assist with research into osteoporosis and osteoarthritis. The zygosity of the twins was determined using a validated questionnaire and in doubtful cases this was confirmed using multiplex DNA fingerprinting. Zygosity testing was performed at the Institute of Molecular Medicine using three polymorphic markers: VNTRs of the apolipoprotein B and heavy chain immunoglobulin genes, and a chromosome 16 microsatellite (D16S292). Probability of zygosity was calculated by sequential analysis, with this method providing accuracy of > 99%. All subjects also completed a detailed nurse-administered risk factor questionnaire (Appendix A).

II.2.2 Population study

Clinical data and biological samples were utilised from women who were participating in the Chingford Study. This study was established in 1988 under the guidance of Dr. Tim Spector and Dr. David Doyle to study the longitudinal epidemiology of osteoarthritis and osteoporosis. In 1988 women in the age range 45-64 had been selected from a large single general practice in Chingford, North-East London (total of 11,000 registered patients) to participate in a longitudinal epidemiological study

of rheumatic diseases. 1,353 women were found to be in the age range specified, and of these 78% (1,003) agreed to participate. The area is predominantly middle class, 98% are white and the population similar to United Kingdom normals in terms of height. weight, smoking status, hysterectomy rates and use of HRT. Fasting blood and urine samples were obtained on all women at each visit and stored at -20° until required for analysis. All women also completed a questionnaire designed specifically to assess risk factors for osteoporosis and osteoarthritis (Appendix B). Subjects have been reviewed annually by the same observer (Dr. Debbie Hart).

II.2.3 Definition of menopausal status

In both studies, premenopausal status was defined as a subject with regular menstruation. If hysterectomy without oopherectomy had been performed this was confirmed by measurement of serum sex hormones – FSH, LH and oestradiol. Postmenopausal status was defined as absence of menstruation for ≥ 12 months and was confirmed in all cases by measurement of female sex hormones, with increases in FSH and LH, and a serum oestradiol < 100 pmol/l.

II.2.4 Consent

Women in both the twin and population based studies gave their informed consent to participate in these studies, and the relevant study protocols were approved by the Local Ethical Committee.

II.3 Bone Mineral Density Measurements

II.3.1 Twins

BMD was measured using DXA with a Hologic QDR-2000 (Hologic Inc., Waltham, MA). Measurements were taken at the lumbar spine (L1-L4), hip (femoral neck, Ward's triangle, total hip and inter-trochanteric regions), forearm (3 regions: ultradistal, distal and mid-third) and total body BMC.

II.3.2 Population study

In the Chingford Study, BMD was measured using DXA with a Hologic QDR-1000. Annual measurements had been taken at the lumbar spine and femoral neck over a six-year period in all women.

II.3.3 Reproducibility

Daily quality control for the Hologic machines was performed using standard spine phantoms. Reproducibility *in vivo* was assessed with duplicate measures in 12 healthy female volunteers. The coefficient of variation was 0.8 % at the lumbar spine, 1.4% at the femoral neck, at the forearm and total body.

II.3.4 Definition of osteoporosis

Based on WHO disease criteria (WHO, 1994), subjects were classified as having osteoporosis at either the spine or hip if their BMD was less than 2.5 standard deviations below the mean peak young adulthood value for that site. The Hologic database was used as the reference range for this calculation.

II.3.5 Rates of change in BMD

Annual measurements of BMD were available on women from the population cohort. In calculating the rate of change in BMD, it was assumed that the expected change in BMD was linear with time for each subject with variation in slopes and intercepts from subject to subject. The deviations of the measured BMD for a subject from the expected BMD were expected to have a zero mean and constant variance and to be uncorrelated. Under these assumptions a linear regression equation, where BMD is a dependent variable expressed as a linear function with time (in years), was fitted for individual subjects. Under this model, the annual percentage change in BMD for each subject was then derived by dividing the regression slope by the intercept at time zero. If hormonal treatment or other treatments known to affect bone metabolism such as steroids, bisphosphonates, calcium or vitamin D was initiated during the study period, then BMD data were included only up to that time point.

II.4 Assessment of osteoporotic fractures

II.4.1 Peripheral fractures

At their initial examination, all women from the Chingford population study reported their personal history of fracture for the 10-year period preceding the study onset (1978-88). Because of the cohort's age range (45-64 years) all reported fractures had been sustained after the attainment of peak bone mass. Osteoporotic fractures sustained by these women were defined as fractures at the wrist and hip that had occurred after minimal trauma. The circumstances under which the fracture had occurred were detailed in a postal questionnaire. Major traumatic fractures were classified if they had occurred following a road traffic accident, a fall from the height of a chair or greater, or a fall down a flight of stairs. Peripheral fractures in these subjects had been previously validated by examination of the subjects' medical records (Arden et al, 1996b).

II.4.2 Vertebral fractures

At their baseline assessment all 1003 women underwent radiological survey of their thoracic (T4-T12) and lumbar (L1-L4) spines using standardised procedures. Spine fractures, which are mostly asymptomatic, were assumed to be osteoporotic in origin. Fractures were defined with morphometric analysis using a semi-automated digitiser and a validated algorithm (McCloskey et al, 1993). Fracture was defined as at least two 2 SD deformities or one 3 SD deformity of either anterior or posterior vertebral body height. These criteria have been shown to be associated with low BMD in population studies and are equivalent to more stringent cut-offs used by other groups (Eastell et al, 1991; Black et al, 1991).

II.5 Molecular studies

II.5.1 General Reagents

Molecular biology and analytical grade chemical reagents were obtained from the following suppliers: BioRad Ltd (Richmond CA, USA), International Biotechnologies Inc (IBI; New Haven, CT, USA), and the Sigma Chemical Co (Louis, MO, USA). Enzymes for molecular biology were purchased from Boehringer Manheim (Manheim, W Germany), Pharmacia LKB Biotechnology (Uppsala, Sweden) or from Promega Corporation (Maddison, WI, USA).

II.5.2 Solutions and buffers

(i) Tris saturated phenol: liquefied phenol saturated with 0.1M Tris-HCL pH 8.0.

(ii) TBE: 50mM Tris-HCl, 40mM boric acid, 1mM EDTA, pH 8.0

(iii) TE: 10mM Tris-HCl, 1mM EDTA, pH 8.0

(iv) Red cell lysis buffer: 15mM NH_4CL, 10mM $NaHCO_3$, 1mM EDTA, pH 8.0

(v) Leucocyte lysis buffer: 10mM Tris-HCl, 0.2% SDS, 50mM EDTA, pH 8.0

II.5.3 Genomic DNA extraction

Peripheral whole blood was taken into EDTA tubes (2 x 10ml). Tubes were centrifuged for 10 minutes in a bench top centrifuge. The buffy coats were collected, pooled and resuspended in normal saline (0.9 % NaCl) and centrifuged as above to minimise red cell contamination. The washed, pooled buffy coat was resuspended in red cell lysis buffer at 4°C for 20 minutes to lyse any residual red blood cells. The intact

leucocytes were then centrifuged again as above and the pellet resuspended in 1ml of normal saline. 2ml of leucocyte lysis buffer were then added to lyse the leucocytes producing a viscous crude lysate. This crude preparation could be stored at -20°C prior to the DNA extraction process if necessary.

DNA extraction utilised a phenol/chloroform method. Briefly, to each crude leucocyte lysate was added 50 µl (10mg/ml) of proteinase K (Boehringer Manheim, W. Germany) and 250 µl 10% SDS. Each sample was incubated at either 65°C for 1-4 hours or at 37°C overnight. 1.5ml of equilibrated phenol and 1.5ml of 24:1 chloroform/isoamyl were added to each sample. Samples were then mixed, centrifuged and upper phase transferred to a clean tube. This extraction process was repeated. Then 3ml of chloroform/isoamyl (24:1) were added to the pooled upper phase that was again centrifuged before the upper phase was again transferred to a clean tube. Adding 2.5 volumes of 100% ethanol to the pooled upper phases precipitated DNA and tubes were then placed at -20°C for 1 hour. The precipitate was removed by centrifugation, washed with 70% ethanol, and subsequently air dried prior to dissolving in 1ml of TE. DNA concentration and purity was measured by absorbane at 260 and 280 nanometers, with readings of 1.6 to 1.8 indicating that the DNA was of sufficient purity for subsequent analysis.

II.5.4 The polymerase chain reaction

The PCR exploits certain features of DNA replication. DNA polymerase uses single-stranded DNA as a template for the synthesis of a complementary new strand. This single strand DNA template can be produced by heating double stranded DNA (dsDNA) to temperatures near boiling. DNA polymerase also requires a small section of ds-DNA to initiate ("prime") synthesis, and the starting point for the DNA synthesis can therefore be specified by supplying an oligonucleotide primer that anneals to the template and that specific point. Both DNA strands can serve as templates for synthesis provided that an oligonucleotide primer is supplied for each strand. The net result of a PCR is, therefore, that by the end of n cycles the reaction will contain a theoretical maximum of 2^n ds-DNA molecules that are copies of the DNA sequence between the oligonucleotide primers.

The starting material for PCR is genomic DNA. To this are added DNA polymerase, specific oligonucleotide primers, and a mixture of all four deoxynucleotide (dNTP) precursors. The reaction mixture then undergoes a series of cyclical temperature changes to amplify the DNA region of interest (Figure II.1). The annealing temperature is the key variable in determining the specificity of the PCR, and temperatures and times used will vary depending on the sequence to be amplified.

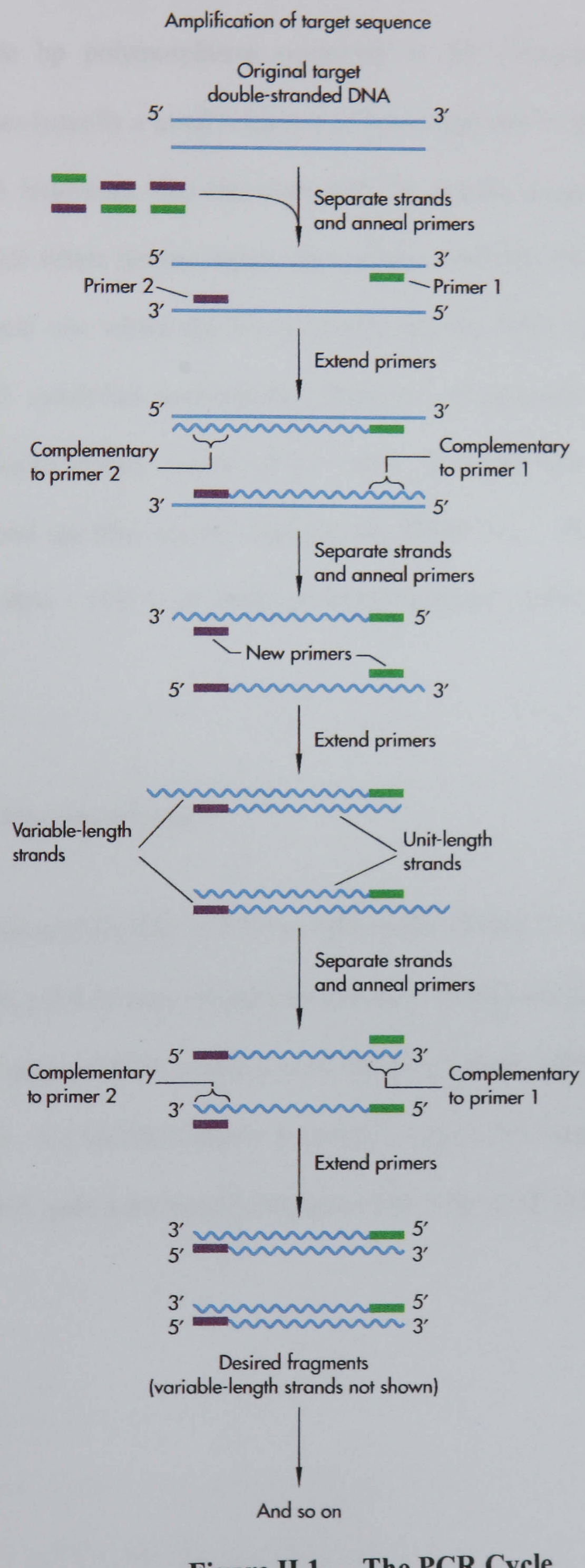


Figure II.1 The PCR Cycle

II.5.5 Restriction fragment length polymorphisms

Single bp polymorphisms occurring at the recognition sites for restriction endonucleases (usually a small sequence of 4 to 6 bp) can be identified by analysis of the size of DNA fragments after digestion with the specific enzyme. A biallelic RFLP will have one allele where the restriction site is present and the DNA is cleaved into 2 smaller fragments, and one where the site is absent and the DNA will not be cleaved. Early studies used restriction endonuclease digestion of genomic DNA with radiolabelled cDNA probes to identify polymorphic variants. PCR has now allowed the amplification of smaller and specific regions flanking the RFLP site. This has allowed the DNA fragments (often < 1kb) to be easily visualised using gel electrophoresis.

II.5.6 Gel Electrophoresis

Agarose (IBI) gels (1-2 %) in TBE buffer (50mM Tris-HCl, 40mM boric acid, 1mM EDTA, pH 8.0) were prepared containing 0.5 μg/ml ethidium bromide. PCR products or digested PCR products were separated using a Biorad gel apparatus at 100 V for 1 hour. A 1 kb DNA marker was used to size DNA fragments for all gels. To visualise DNA, gels were transilluminated under ultraviolet light and photographed.

II.6 Detection of candidate genes using PCR

II.6.1 Vitamin D receptor gene

II.6.1.1 TaqI genotype

Oligonucleotides

Primers sequences 5'-CAG AGC ATG GAC AGGGAG CAA G-3' and 5`-GCA ACT CCT CAT GGC TGA GGT CTC A-3' producing a 740 bp fragment.

PCR conditions

Reactions were performed in 20 µl containing 200 ng of genomic DNA, 20 pmol of each primer, 200 µM dNTPs, 50mM KCL, 10mM Tris (pH 9.0 at 25°C), 0.1% Triton® X-100, 2.0mM $MgCl_2$ and 1 unit Taq DNA polymerase (Promega Corporation). Each sample was subjected to an amplification cycle in a Corbett Research FTS-1 Thermocycler (Corbett Research, NSW, Australia) as follows:

Step1: 3 min at 94°, 1 min at 62°, 2 min at 72°.

Steps 2-6: 20 sec at 94°, 20 sec at 62°, 1 min at 72°.

Steps 7-47: 5 sec at 94°, 5 sec at 62°, 30 sec at 72°.

Step 48: 3 min at 72°.

Restriction digest conditions

The restriction endonuclease *TaqI* recognises the sequence $T^{\downarrow}CGAA$. A 10 µl aliquot of each PCR product was digested with 5 units of *TaqI* (Promega Corps) in a total volume of 20 µl (1mM Tris-HCl [pH 7.4], 30mM KCl, 10µM EDTA, 100µM

DTT, 0.5mg/ml BSA, 5% glycerol) at 65° for 1 hour. The invariant *TaqI* site in the PCR product acted as an internal control for the enzyme digest. The digested products were then separated using gel electrophoresis and a 2% agarose gel.

Allele designation for the *TaqI* RFLP associated with VDR

The banding pattern for the *TaqI* restriction enzyme is shown in Figure II.2. The alleles were coded as "T" and "t", where the upper case signifies absence of the polymorphic restriction site and the lower case presence of the site. In homozygous absence of the *TaqI* site (TT) the enzyme digestion of the 740 bp PCR product at the invariant *TaqI* site yields 245 bp and 495 bp bands. Homozygous presence of the *TaqI* site (tt) yields fragments of 205 bp, 245 bp and 290 bp. Heterozygotes for the *TaqI* RFLP (Tt) exhibit fragments at 490 bp, 290 bp, 245 bp and 205 bp.

II.6.1.2 FokI genotype

Oligonucleotides

Primer sequences 5'-GAT GCC AGC TGG CCC TGG CAC TG-3' and 5'-ATG GAA ACA CCT TGC TTC TTC TCC CTC-3' producing a 272 bp PCR product.

PCR conditions

Reactions were performed in a 20 µl volume, containing 200 ng of genomic DNA, 20 pmol of each primer, 200 µM dNTPs, 50mM KCL, 10mM Tris (pH 9.0), 2.0mM $MgCl_2$ and 1 unit Taq DNA polymerase (Promega Corporation). Each sample was subjected to an amplification cycle in a Corbett Research FTS-1 Thermocycler (Corbett Research, NSW, Australia) as follows:

Step1:	3 min at 95°, 1 min at 65°, 1 min at 74°.
Steps 2-6:	20 sec at 95°, 30 sec at 65°, 30 sec at 74°.
Steps 7-37:	5 sec at 95°, 5 sec at 65°, 15 sec at 74°.
Step 38:	3 min at 74°.

Restriction digest conditions

A 10 µl aliquot of each PCR product was digested with 5 units of *FokI* (Promega Corporation) in a total volume of 20µl (1mM Tris-HCl [pH 7.4], 5mM NaCl. 10µM EDTA, 100µM DTT, 0.5mg/ml BSA, 5% glycerol) at 37° for 3 hours. The digested products were then separated using gel electrophoresis and a 2% agarose gel.

Allele designation for the *FokI* RFLP associated with VDR

The restriction endonuclease *FokI* recognises the sequence GGATG, and therefore detects the presence of the ATG sequence. The absence of the *FokI* site, indicated as "F", therefore demonstrates that the first ATG site is absent. Presence of the *FokI* site, indicate by "f", will result in cleavage of the 272 bp PCR product by the restriction endonuclease to yield two fragments of 198 bp and 74 bp. Individuals homozygous for the FF genotype exhibit a single uncut fragment of 272 bp, while homozygotes for the ff genotype have two fragments of 198 and 72 bp. The heterozygote Ff genotype has all three bands (Figure II.3).

Figure II.2 **Allele banding pattern for the *TaqI* VDR RFLP**

M = marker for sizing C = undigested PCR product

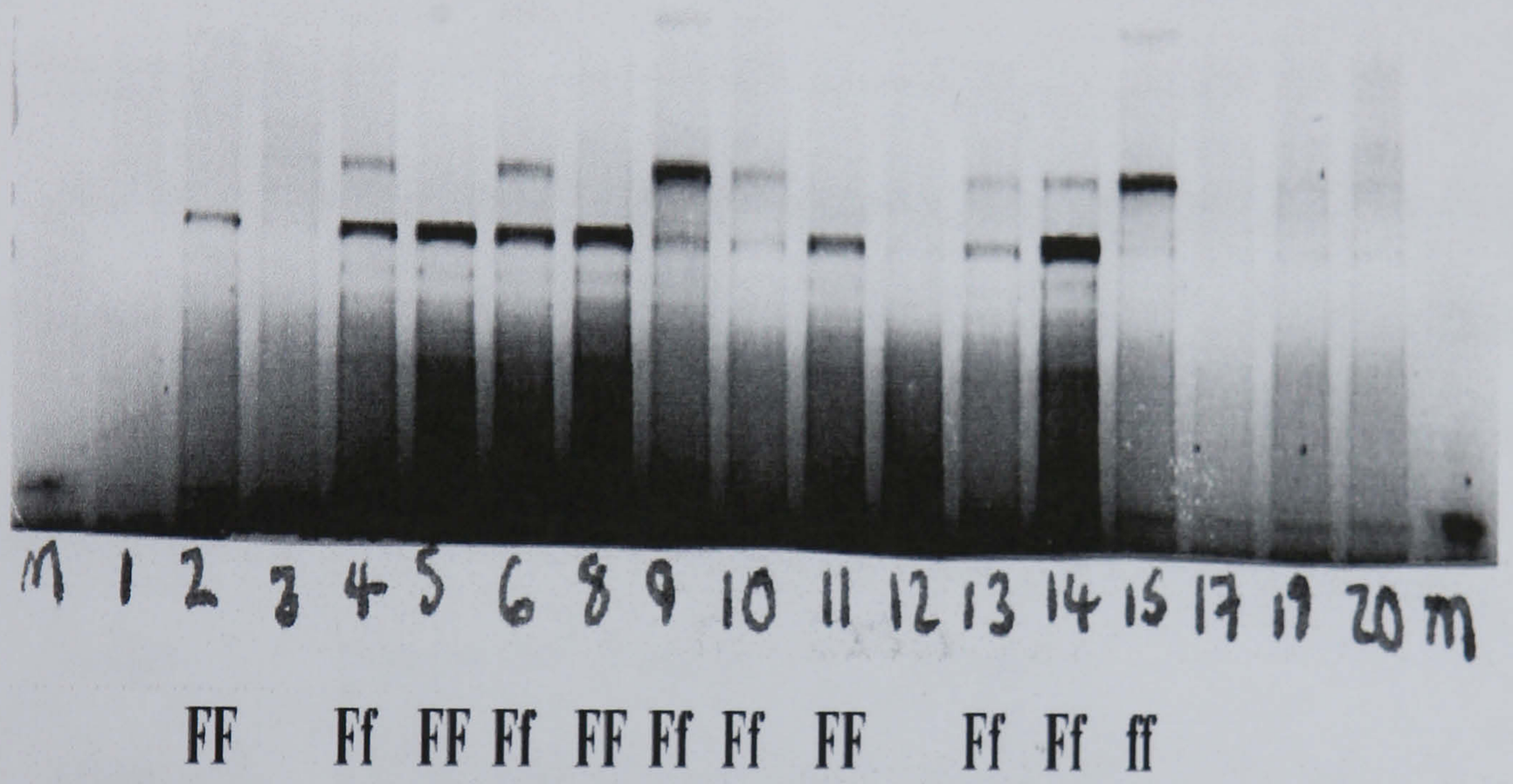


Figure II.3 **Allele banding pattern for the *FokI* VDR RFLP**

Lanes 2, 5, 8, 11 = genotype FF

Lanes 4, 6, 9, 10, 13, 14 = genotype Ff

Lane 15 = genotype ff

M = marker for sizing

II.6.2 Oestrogen receptor gene

Oligonucleotides

Primers sequences 5'-CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC TCC-3' and 5'-TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA-3' producing a 1.3 kb PCR product.

PCR Conditions

Reactions were performed in 20 μl containing 200 ng of genomic DNA, 20 pmol of each primer, 200 μM dNTPs, 50mM KCL, 10mM Tris (pH 8.3), 2.0mM $MgCl_2$ and 5 units Taq DNA polymerase (Promega Corporation). Each sample was subjected to an amplification cycle in a Corbett Research FTS-1 Thermocycler as follows:

Step1: 5 min at 94°, 1 min at 64°, 1 min at 72°.

Steps 2-31: 20 sec at 94°, 20 sec at 62°, 1 min at 72°.

Step 32: 3 min at 72°.

Restriction digests

A 10 μl aliquot of each PCR product was digested with either 5 units of *PvuII* or *XbaI* (both Promega Corps) at 37° for 1 hour. The digested products were then separated using gel electrophoresis and a 1.2% agarose gel.

Allele designation for RFLPs associated with ERα

Coding for ERα gene alleles uses a similar nomenclature to that used for the VDR gene, such that the upper case letter signifies absence of the polymorphic restriction site and the lower case letter presence of the site. The banding pattern for the

PvuII restriction enzyme is shown in Figure II.4. The "p" allele produced fragments of 0.85 kb and 0.45 kb. A similar pattern was also observed for the *XbaI* genotypes, with the "x" allele having fragment sizes of 0.9 kb and 0.4 kb.

II.6.3 Type I Collagen α1 gene

Oligonucleotides

Primer sequences 5'-TAA CTT CTG GAC TAT TTG CGC ACT TTT TGG-3' and 5'-GTC CAG CCC TCA TCC TGG CC-3' were utilised to amplify a region within the first intron of the COLIA1 gene. The underlined bases in the reverse primer represent mismatched bases that introduce a *BalI* restriction site in polymorphic alleles with the G→T substitution.

PCR Conditions

PCR reactions were performed in 25 μl containing 200 ng of genomic DNA, 20 pmol of each primer, 200 μM dNTPs, 50mM KCL, 10mM Tris (pH 8.3), 1.5mM $MgCl_2$ and 5 units Taq DNA polymerase (Promega Corporation). Each sample was subjected to an amplification cycle in a Perkin Elmer 480 Thermocycler (Perkin Elmer, Foster City, CA, USA) to produce a 264 bp product as follows:

Step 1:	3 min at 94°
Steps 2-36:	1 min at 94°, 1 min at 60°, 1 min at 72°
Step 37:	3 min at 72°

Restriction digests

A 10 µl aliquot of each PCR product was digested with 5 units of *MscI* (Promega Corps) in a buffer containing 150 mM TRIS-HCl (pH 7.5), 250 mM NaCl and 35 mM $MgCl_2$ at 37° overnight. *MscI* is an isoschizomer of *BalI*, with both enzymes recognising the sequence TGG↓CCA. The digested products were then separated using gel electrophoresis and a 3% agarose gel.

Allele designation for RFLPs associated with COLIA1

Alleles were coded as "S" for presence of the guanine residue and "s" for the rarer thymidine residue. The "s" allele resulted in two fragments of 246 bp and 18 bp, although the latter was not detectable with a 3% agarose gel. The "S" allele represented non-digestion of the PCR product as was of size 264 bp. Banding patterns for the COLIA1 polymorphism are shown in Figure II.5.

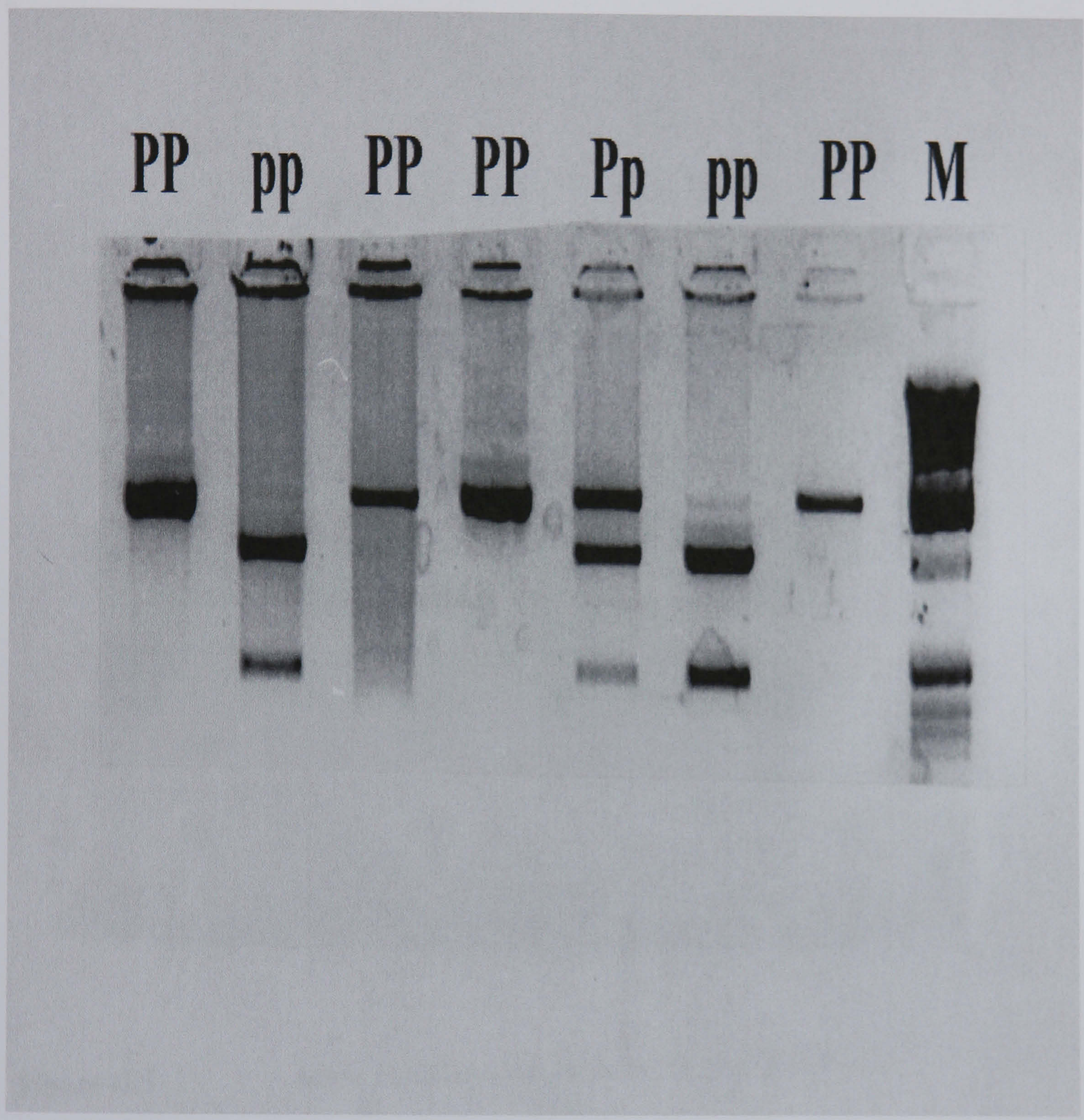


Figure II.4 **Allele banding pattern for the *PvuII* ER RFLP**

M = marker for sizing

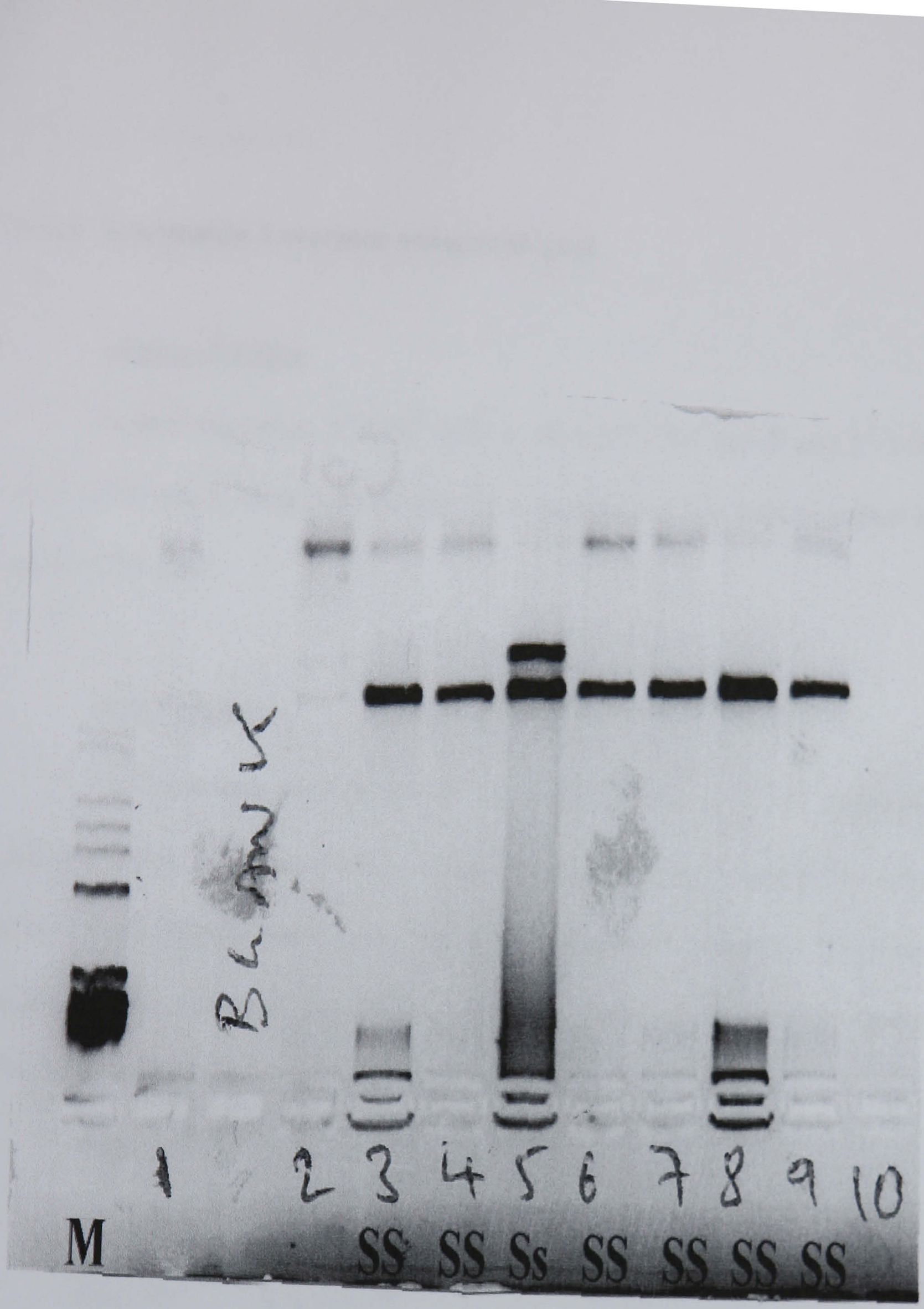


Figure II.5 **Allele banding pattern for COLIA1 RFLP**

Lanes 3, 4, 6, 7, 8, 9 = genotype SS

Lane 5 = genotype Ss

M = marker for sizing

II.6.4 Interleukin 1 receptor antagonist gene

Oligonucleotides

Primer sequences 5'-CTC AGC AAC ACT CCT AT-3' and 5'-TCC TGG TCT GCA GGT AA-3' were used to amplify a variable number tandem repeat in intron 2 of the IL-1RN gene.

PCR Conditions

PCR reactions were performed in a total volume of 20 μl, containing 200 ng of genomic DNA, 20 pmol of each primer, 200 μM dNTPs, 50mM KCL, 10mM Tris (pH 8.3), 5.0mM $MgCl_2$ and 1 unit Taq DNA polymerase (Promega Corporation). Each sample was subjected to an amplification cycle in a Corbett Research FTS-1 Thermocycler as follows:

Step 1:	1 min at 96°
Steps 2-31:	1 min at 94°, 1 min at 60°, 2 min at 70°
Step 32:	3 min at 72°

Allele designation for RFLPs associated with IL1RN

The final PCR products were analysed on 1.2% agarose gels. Previous work in 70 unrelated individuals (Tarlow et al, 1993) had identified 5 alleles at this locus. Alleles were classified according to their relative size on gel electrophoresis (Figure II.6).

II.6.5 Quality control

All PCR reactions contained a negative control that contained no DNA. This was to ensure that all the PCR ingredients were not contaminated with exogenous DNA. In addition, all "runs" included samples of known genotype to ensure consistency of the endonuclease digestion. 5-10 % of samples were repeated for internal consistency.

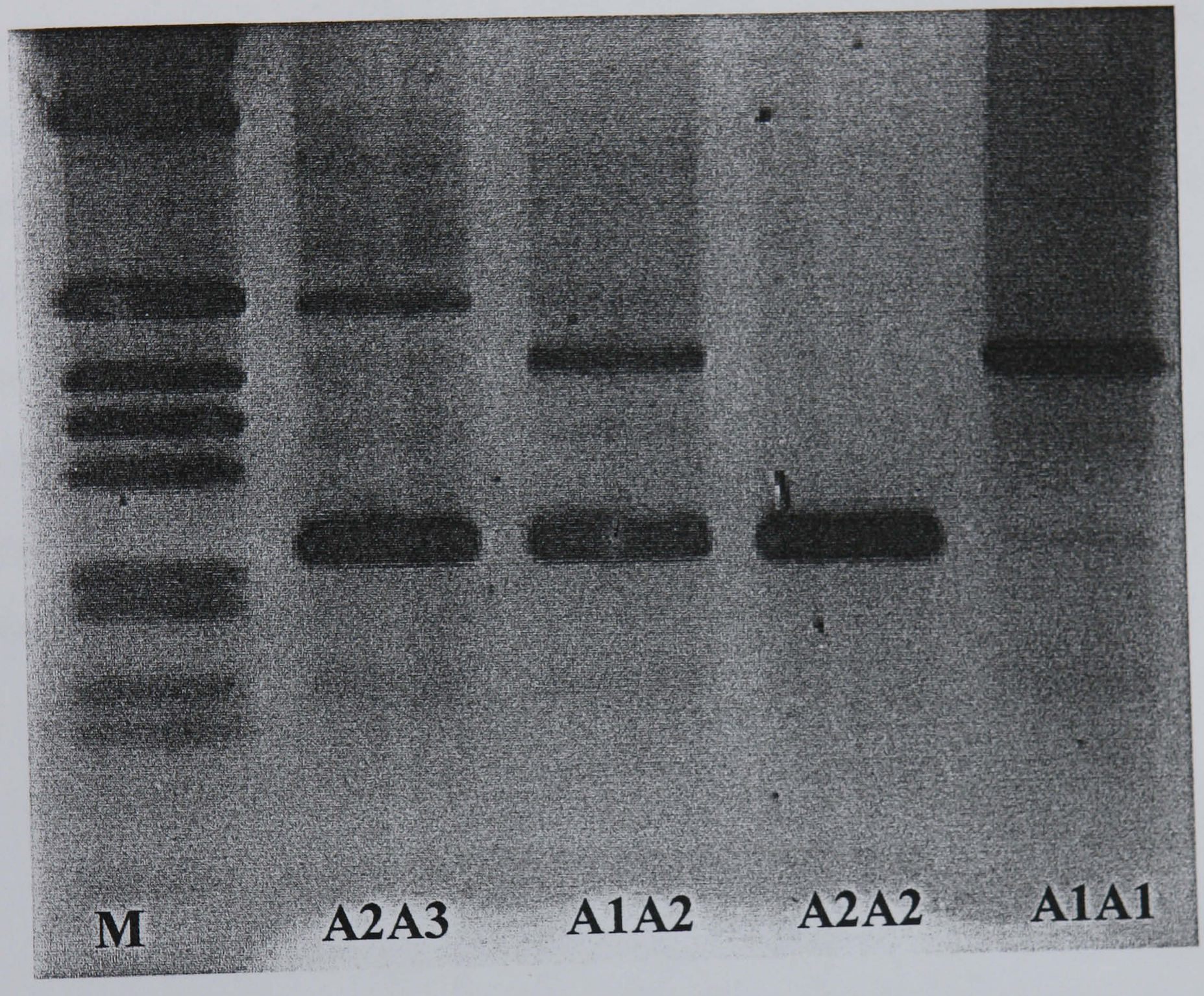


Figure II.6 Allele banding pattern for IL-1RN VNTR

M = marker for sizing

II.7 Statistical Analysis

II.7.1 Heritability analysis

Heritability is defined as a ratio of variances (V), specifically, the ratio of the genetic variance (i.e. that attributable to genotypic or allelic differences among individuals) to the total phenotypic variance in the population. The observed phenotypic population variance (Vp) is partitioned into components reflecting the differences in unobserved genetic (g) and environmental factors (c = common, e = unique), both of which must be estimated from covariances or correlations among relatives. This approach assumes a linear model where the trait is a linear function of genetic and environmental factors. If the genotypes and environment are independent, the total variance of the trait can be defined as:

$$Vp = Vg + Vc + Ve$$

From this breakdown, broad sense heritability can be written as: $h^2 = Vg/Vp$

The classical twin model assumes that MZ twins share 100% of their genes, and therefore any intrapair variations in a measured trait may be due to environmental factors. DZ twins are no more alike than full sibs and share on average 50% of their genes, hence their intrapair variations must be due to both environmental and genetic factors. The twin model also assumes that (1) both sets of twins have been sampled from the same gene pool, (2) that environmental sharing is similar for both MZ and DZ twins, and (3) that the variance due to epistasis is negligible. Under these assumptions a comparison of the correlation between MZ and DZ twins provides an estimate of the relative contribution of genetic factors in the total variation of a quantitative trait. To

statistically estimate this component of genetic variance, the total variance of a quantitative trait is partitioned into two components: one due to within (intrapair, W) variation and one due to between (interpair, B) variation by performing analysis of variance (ANOVA). Intraclass correlations for MZ pairs and DZ pairs are then estimated:

$$r = (B-W)/(B+W).$$

Heritability estimates were calculated using the computer programme TWINAN90 (Williams et al, 1992). The broad sense heritability estimate was calculated as twice the difference of the MZ and DZ intraclass correlations [i.e. 2(rMZ-rDZ)] (Falconer, 1960).

Because co-twins in the same pair tend to share environment as well as genes, heritability estimates could be spuriously increased. This is particularly true if the environmental sharing is greater within MZ twin pairs than it is within DZ pairs. To avoid this potential bias of differential environmental covariance, trait values were adjusted for environmental variables by means of multiple regression analysis. Separate equations were developed for MZ and DZ twins to allow for the potential differential effects of the environmental variables in the two groups of twins. On the basis of these equations a predicted value was calculated for each trait value. A residual value was calculated as the difference between the observed and predicted trait values, and these standardised residuals were then used in the adjusted heritability analysis.

II.7.2 Modified linkage analysis

To assess the contribution of candidate gene polymorphisms on BMD within the DZ twins, modified sib-pair linkage analysis was performed. Intra-class correlations in BMD were calculated using the programme TWINAN90, as described previously, for DZ twins concordant or discordant for genotype. Results were deemed suggestive of linkage at the trait locus if the $rDZ_{concordant}$ was significantly greater than the $rDZ_{discordant}$. Adjustment for potential confounders was also performed as previously described for the heritability analysis.

Classical sib-pair analysis determines whether each sib pair shares 0,1 or 2 alleles identical by descent (IBD) at a locus of interest. For a quantitative trait, sib pairs should show a correlation between the magnitude of their phenotypic difference and the number of alleles shared IBD (Haseman and Elston, 1972). Modifications to this classical method of analysis were required for several reasons. Firstly, parental genotype information was not available for the DZ twins and it was not possible to accurately infer their IBD status. Analysis was therefore based on identical by state (IBS) sharing that is less powerful than IBD analysis (Bishop and Williamson, 1990). As the candidate gene polymorphisms examined were biallelic, DZ twins were simply classified as being either concordant or discordant for their IBS sharing. Secondly, the original report by Haseman and Elston (1972) examined the squared intra-pair difference in trait values, regressing this value against the number of alleles shared IBD. Squaring of the intra-pair difference can, however, magnify errors due to extreme outlying values, and use of crude values provides a more reliable estimate. As the quantitative trait of interest in this

analysis was BMD, which is known to be affected by environmental factors, the use of the TWINAN90 programme to calculate the $rDZ_{concordant/discordant}$ values also allowed adjustment to be made for these potential confounders.

II.7.3 Testing for Hardy Weinberg equilibrium

In a large random-mating population with no selection, mutation or migration, the gene frequencies and the genotype frequencies are constant from generation to generation. A population with such constant gene and genotype frequencies is described as being in Hardy Weinberg equilibrium (Hardy, 1908; Weinberg, 1908). For a gene with two alleles with frequency p and q (1-p), Hardy Weinberg equilibrium (HWE) is maintained if:

$$p^2 + 2pq + q^2 = 1$$

Testing for HWE was made using Chi-squared analysis, comparing the observed genotype frequencies against those expected under random mating.

II.7.4 Association analysis

II.7.4.1 Analysis of variance

Risk factors for quantitative traits cannot cause the trait but they can affect its mean value and variation. It is possible to model the effect of risk factor x_i to individual j's phenotype ϕ_j, by a regression coefficient β_i through an equation such as:

$$\phi_j = \alpha + \sum \beta_i x_{ij}$$

In the simplest genetic case, known as a "measured genotype approach", non-genetic variables are treated as nuisance variables with an additive action on the phenotype, and regression is used to adjust the trait value for these variables. Genotypic risk factors can be treated as qualitative data, set to 1 if an individual has a particular genotype and zero if otherwise. After adjustment, the deviation that remains is what is hoped to be explained by the genotype, and represents a categorisation of the data that can be analysed using analysis of variance (ANOVA).

II.7.4.2 Analysis of trait means

Knowledge of genotype information allows the study population to be grouped according to their underlying genotype. It is possible to test one's data against the null hypothesis that there is no difference in the trait means (μ) of the genotypes using

Student's t-test. For a biallelic locus with two alleles (1/2) the null hypothesis (H_0) would be:

$$H_0: \mu_{11} = \mu_{12} = \mu_{22}$$

At loci with multiple alleles several issues need to be considered. Unless datasets are large the number of individuals in each genotype group will be small and this may reduce the power to identify a difference in mean trait values across the groups. Genotype groups may also be pooled to allow testing of differences in trait means associated with carriage versus non-carriage of a particular allele under both dominant and recessive inheritance models. In these circumstances there may be a large number of potential groupings and comparisons made, and at present it is not clear if statistical adjustments for these multiple comparisons in the form of the Bonferroni correction factor is necessary or appropriate (Altshuler et al, 1998; Perneger 1998).

II.7.4.3 Odds ratios

In a case control study, the frequency of the different genotypes is compared between individuals who have the outcome measure of interest (cases) and a sample of individuals who do not (controls). Measures of odds ratio (OR) can be calculated as follows and closely approximate those of relative risk obtained from cohort studies when the disease or outcome measure is rare (Rothman 1986).

Table II.1 Calculation of the odds ratio from a case-control study

Genotype	Frequency in cases	Frequency in controls	Odds ratio
NN	A_1	B_1	1
NS	A_2	B_2	A_2B_1/A_1B_2
SS	A_3	B_3	A_3B_1/A_1B_3

N: normal allele S: susceptibility allele

The OR equals unity when there is no association between the exposure and the disease. If the OR is greater than unity, then the rate of the disease amongst the exposed is greater than non-exposed. If however the OR is below unity then the rate of disease in the unexposed group is greater. The contingency tables outlined above can accommodate a variety of data stratifications and other complications.

Confidence limits of the OR are used to represent the statistical uncertainty involved and give an impression as to the magnitude of the effect. This aids differentiation between statistical and scientific significance. To calculate the confidence interval the standard error of the OR has to be calculated using maximum likelihood estimation. The 95% confidence interval incorporates the range of values from mean - (1.96xSE) to mean + (1.96xSE).

II.7.4.4 Analysis of confounding

Confounding can occur when an association between a risk factor and disease can be explained by a factor associated with both the disease and the risk factor (Miettinen and Cook, 1981). A confounder must satisfy the following conditions: i) that it is associated with both the study disease and the exposure factor under study, and ii) that the confounder is extraneous to the two main variables (disease and exposure) but can distort their relation. A factor cannot be considered a confounder if it lies on the causal pathway between exposure and outcome or if it determines the presence of an exposure variable but has no direct effect on outcome. In the analysis of data, confounding can be seen when the OR is altered after adjustment for another factor.

Confounding can be handled in the analysis by matching, stratification and/or use of multivariate models. Stratification provides a direct way by which biased comparisons due to confounding can be eliminated. The major limitation of stratification is, however, the number of strata that can be analysed simultaneously, as the greater the number of strata the less the numbers of subjects in each cell. Schlesselman (1982) has estimated that 1280 cases and 1280 controls would be needed to examine the joint effects of 7 dichotomous variables with stratification. Logistic regression modelling can be expanded to include the effects of quantitative and qualitative variables simultaneously. Dose response relationships can be defined using a linear regression model:

$$y = ax + b$$

where y is the disease probability, x the exposure variable, and a and b are parameters to be estimated. This model assumes, however, a linear relationship between the probability

of disease and the exposure variable. Logistic regression models the relationship between disease probability and exposure as linear on a logit scale:

$$y = \frac{\exp(a + bx)}{1 + (\exp(a + bx)}$$

The above equation can also be expanded to include several variables simultaneously:

$$\ln \frac{P(Y = 1)}{1 - P(Y = 1)} = a + b_1x_1 + b_2x_2 + ... + b_nx_n$$

where x_1, x_2,...x_n represent n independent variables used to predict the probability of disease.

Values for the regression coefficients b_i can be obtained using maximum likelihood procedures and these can be interpreted in terms of the OR, computed as OR = exp (b_n), for each risk factor after adjustment for the effects of other variables.

II.7.4.5 Interaction analysis

Interaction or effect modification has to be considered as it can be influenced greatly by the process of adjustment for confounding. If the impact of a risk factor on the outcome varies across different values of a third variable, then "statistical interaction" or effect "modification exists". This third variable could be either an environmental exposure (i.e. dietary calcium intake) or an independent genetic marker. Although methods exist for dealing with interaction in logistic regression (Kelsey et al. 1986),

simple adjustment for such a third variable may mask important biological relationships. Stratification may reveal useful information (Ottman 1990) although, as discussed, large sample sizes would be required. In many instances the models are simplistic using a biallelic gene polymorphism at a single locus and a single dichotomous environmental exposure (present vs absent).

II.7.4.6 Sensitivity and specificity

Measuring commonly assesses the accuracy of a diagnostic procedure as follows:

1) sensitivity (the proportion of true positives correctly identified)

2) specificity (the proportion of true negatives correctly identified).

These concepts can be applied to various methods of ascertainment such as the reporting of a positive family history for disease or carriage of a disease risk genotype.

Table II.2 Sensitivity and specificity

	Disease status	
Test status	+	-
+	a	b
-	c	d

Sensitivity (se) = a/(a + c) Specificity (sp) = d/(b + d)

II.7.4.7 Positive and negative predictive values

Another important concept is the predictive value of a test. The positive predictive value (PPV) refers to the fraction of persons classified as having the disease or trait by the test who actually do have the trait. The negative predictive value (NPV) refers to the fraction of subjects classified as not having the disease by the test whom actually do not have the trait. PPV and NPV are functions of sensitivity, specificity, and the frequency (prevalence) of the underlying trait in the population tested. The rarer the trait, the lower the PPV will be for any given level of sensitivity and specificity. Unless the test is both 100% sensitive and 100% specific there will always be some misclassification.

$$PPV = P(\text{disease} + \mid \text{test} +)$$

$$= \frac{(se) \times (g)}{(se) \times (g) + (1 - sp)(1 - g)}$$

$$NPV = P(\text{disease} - \mid \text{test} -)$$

$$= \frac{(sp) \times (1 - g)}{(1 - se) \times (g) + (sp) \times (1 - g)}$$

where g is the frequency of the disease or trait in the population.

II.7.5 Hardware and software

The data were entered on an IBM compatible 880-Megabyte hard disc computer. The basic statistical calculations including frequencies, tabulations, ANOVA and Student's t-test were all performed using the statistical programme STATA (Stata Corporation, TX, USA). Intra-class correlations for the MZ and DZ twins were computed using the analysis programme TWINAN90 (Williams et al, 1992).

CHAPTER III

FAMILIAL AGGREGATION OF BONE MINERAL DENSITY AND OSTEOPOROTIC FRACTURE RISK

III.1 Summary

In this chapter data have been examined to determine the genetic component to BMD and fracture risk in women from the UK population.

A classical twin analysis was performed to assess the genetic contribution to BMD at multiple sites (lumbar spine, hip, forearm and total body) in 87 MZ pairs and 95 DZ postmenopausal twin pairs. Within-pair correlations in BMD were highest in the MZ pairs compared to the DZ pairs at all sites, with heritability estimates ranging from 0.60 to 0.98. These findings were independent of confounders such as age, weight, menopause duration, smoking status and HRT use.

Data are also presented from a population-based analysis of unrelated women where familial history of an osteoporotic fracture in a female first-degree relative was used as a surrogate measure of genetic susceptibility. BMD was modestly reduced by 0.4 SD at both the spine and the hip in those with a positive family fracture history, when compared to those with a negative history. Family history of any osteoporotic fracture was also associated with an increased total risk for fracture, and site specific analysis showed that a positive family history of wrist fracture was associated with a four-fold increased risk of fracture at this site. These increases in risk remained after adjustment for BMD, suggesting that other genetic factors may also contribute towards this familial risk associated with osteoporotic fracture.

III.2 Introduction

In Chapter I, data from family and twin studies demonstrated that genetic factors appear to play a central role in the regulation of bone mass. These findings are, however, specific to the populations that have been studied and at the onset of this thesis only limited data were available regarding the genetic contribution to bone mass in women from the UK. There was also little data regarding the familial segregation of fracture, with most studies concentrating primarily on hip fracture risk (Cummings et al. 1995).

In this chapter there were two main aims. Firstly, classical twin analysis was used to study the similarity in bone mass measures between MZ and DZ postmenopausal twins from the UK. This would provide an estimate of the broad heritability for BMD at multiple skeletal sites in the UK population, thereby allowing comparison with other published studies. The second aim was to examine the genetic contribution to fracture risk, although as the number of prevalent fractures in the twin group was too small for accurate analysis a population-based analysis was performed. In this, a positive family history of osteoporotic fracture in female first-degree relatives (i.e. mother and/or sisters) was used as a measure of genetic susceptibility to fracture. In these unrelated women from the general population reported family history was then examined for association with low BMD and fracture risk at multiple sites.

III.3 Methods

III.3.1 Twins

109 white Caucasian female DZ twin pairs were studied. These subjects were aged 50-69 years of age and were healthy and not known to be suffering from any disease affecting bone metabolism. In total, 96 DZ pairs were concordant for postmenopausal status and were included in the analysis. In one pair, one twin suffered from an eating disorder and she and her co-twin were subsequently excluded from the analysis due to gross discordance in their body weight (65 kg vs 142 kg). 97 healthy MZ female pairs were also included in the analysis, of which 87 pairs were concordant for postmenopausal status. BMD measurements at the lumbar spine, hip, forearm and total body were available on all subjects.

III.3.2 Population study

Data were available from all 1003 women participating in the Chingford population study. At their initial assessment all subjects had been asked in the nurse-administered questionnaire about a history of osteoporotic fractures occurring at either hip or wrist in a female first degree relative aged > 35 years. These fractures were self-reported and it was not possible to validate fractures occurring in the subjects' relatives. Women reporting a history of wrist or hip fracture occurring in either a mother and/or sister were classified as having a positive family history for osteoporotic fracture. BMD measurements at the lumbar spine and femoral neck were available on all subjects. Details of prevalent vertebral fractures and validated peripheral fractures for the 10-year

period prior to the study's commencement (1978-1988) were also available on all women.

III.3.3 Statistical analyses

In twin subjects, the intraclass correlations for BMD measures within MZ and DZ pairs were calculated, with heritability estimates derived using the Falconer equation. Adjustment for potential confounders was performed using linear regression.

In the population-based analysis, differences in demographic variables between subjects with a positive family history for fracture and those with a negative history were compared by an unpaired *t*-test for normally distributed variables and by a Mann-Whitney *U*-test for non-parametric variables. Categorical variables were analysed using a $\chi 2$ test. Multivariate analysis was performed using logistic regression to estimate the odds ratio and 95% test based confidence intervals for sustaining a fracture by family history fracture status. The sensitivity, specificity, PPV and NPV for family history of fracture as a predictive tool were also assessed.

III.4 Results

III.4.1 Twin analysis

Full clinical data were available on 87 MZ and 95 DZ pairs. No significant differences in baseline characteristics were observed between the two zygosities (Table III.1), although the MZ twins tended to have lower weights, a reduced use of HRT and a

reduced frequency of smoking. The means and variances for BMD at all skeletal sites were similar for MZ and DZ twins. The intra-class correlations in BMD were significantly higher in the MZ twins compared to the DZ twins at all sites (Figure III.1). with significant Falconer heritability estimates (Table III.2). These findings were all essentially unaltered after adjustment for potential confounders (i.e. age, weight, menopause duration, HRT use, smoking status).

Table III.1 Mean characteristics (SD) of 174 MZ and 190 DZ postmenopausal twins

Variable	MZ twins (n = 174)	DZ twins (n=190)
Age (yrs)	59.7 (4.6)	58.7 (5.5)
Height (cm)	160.2 (5.7)	162.1 (6.2)
Weight (kg)	62.5 (9.2)	65.1 (11.2)
BMI (kg/m^2)	24.4 (3.4)	24.8 (4.4)
Age at menopause (yrs)	49.2 (4.4)	48.9 (4.9)
Years since menopause (yrs)	10.5 (6.4)	9.9 (7.4)
No. ever use of HRT (%)	65 (37)	85 (45)
Median duration of HRT use (months)	24 (6, 48)*	24 (6, 48)*
No. ever smoked (%)	66 (38)	99 (52)

* interquartile range

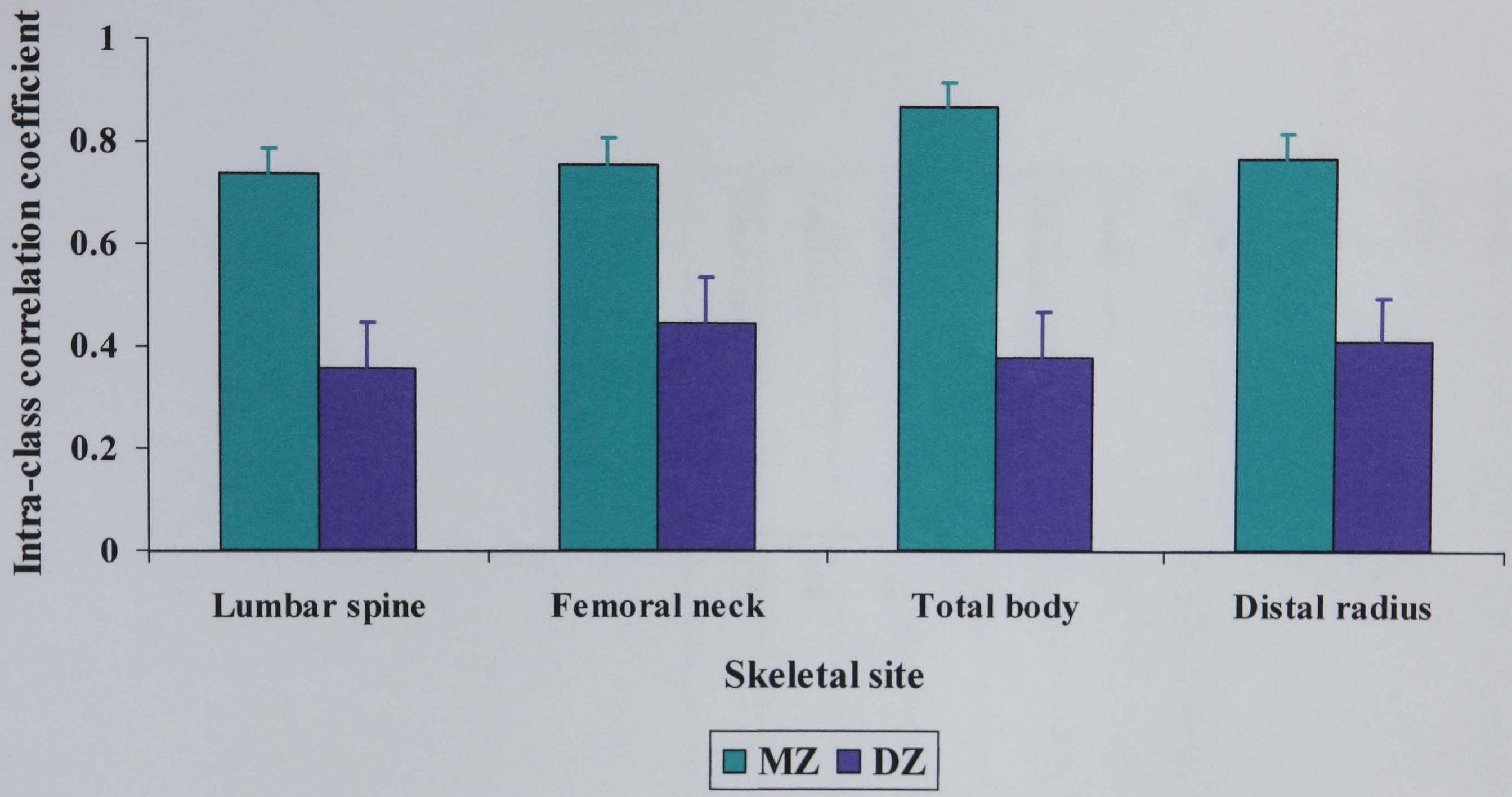


Figure III.1 Intraclass correlation coefficients (± SE) for BMD at various skeletal sites in 87 MZ and 95 DZ pairs

Table III.2 **Falconer heritability estimates for BMD at various skeletal sites from data in 87 MZ and 95 DZ postmenopausal twin pairs**

Site	Heritability estimate
Lumbar spine	0.76
Femoral neck	0.63
Ward's triangle	0.68
Total hip	0.65
Total body	0.98
Distal radius	0.73

III.4.2 Population analysis

Cross-sectional data were available on all 1003 women from the Chingford Study, mean age (SD) of 54.2 (6.0) years. A history of hip fracture in female first-degree relatives was reported in 44 subjects (39 maternal and 5 sibling fractures). At the wrist 107 fractures were reported (83 maternal and 24 sibling), with 6 subjects having a history of fracture at this site in both their mother and a sister. In total, a positive family history of any osteoporotic fracture in either a mother and/or sister was reported in 138 of the 1003 women.

Relationship between family fracture history and BMD

When compared to those with a negative family history of fracture, BMD was significantly reduced in those with a positive history at spine and hip: spine, mean difference (95% CI) of 0.04 g/cm^2 (0, 0.08), p = 0.02, and at the hip, mean difference 0.03 g/cm^2 (0, 0.05), p = 0.02 (Table III.3). Overall, the prevalence of established osteoporosis was 9.9 % at the lumbar spine and 2.5 % at the femoral neck. The risk of a subject having spinal osteoporosis was increased in those with a positive family fracture history when compared to those with a negative history, with an odds ratio (95% CI) of 1.82 (1.08, 3.05), p = 0.02. A similar trend was also seen at the hip although this was non-significant, with an odds ratio of 1.72 (0.63, 4.71), p = 0.29.

Relationship between family fracture history and fracture risk

In total, 119 non-vertebral fractures were reported. After validation and exclusion of fractures due to major trauma there were 51 validated fragility fractures at appendicular sites including 16 at the wrist and 6 at the hip. From the radiological survey of the thoracolumbar spine, 41 prevalent vertebral deformities consistent with fracture were also identified. In total therefore, 60 subjects were found to have validated evidence of prevalent osteoporotic fractures at either the spine, hip or wrist, with one woman having fractures at both hip and spine whilst a further two women had fractures at both the spine and wrist.

Table III.3 Mean characteristics (SD) of 1003 women according to the presence or absence of a family history for osteoporotic fracture (hip and/or wrist) in a female first-degree relative

Variable	Positive Family History (n =138)	Negative Family History (n= 865)	P - value
Age (yrs)	54.2 (6.2)	54.2 (6.0)	0.99
No. postmenopausal subjects (%)	102 (74%)	622 (72%)	0.64
Time past menopause (yrs)	8.0 (5.4)	8.6 (6.0)	0.29
BMI (kg/m^2)	25.4 (4.5)	25.6 (4.3)	0.54
No. ever use of HRT (%)	31 (22%)	207 (24%)	0.70
No. ever smoking (%)	61 (44%)	402 (46%)	0.63
Lumbar spine BMD (g/cm^2)	0.94 (0.16)	0.98 (0.16)	0.02
Femoral Neck BMD (g/cm^2)	0.74 (0.12)	0.77 (0.12)	0.02
No. subjects with osteoporotic fractures (%)	14 (10%)	46 (5%)	0.03

A positive family history of any fracture was associated with an increased total risk for osteoporotic fracture, with adjustment for BMD and other potential confounding variables having little effect on the odds ratio (Table III.4). This increase in risk associated with the positive family history appeared related to appendicular fractures rather than vertebral deformity/fracture, and site-specific analysis showed that a positive family history of wrist fracture was associated with a 4-fold increased risk of wrist fracture (Table III.5). Again, this increase in risk remained significant after adjustment for BMD. There was, however, no significant relationship between a positive family history of wrist fracture and prevalent fracture status at either the spine or hip. There was also no apparent relationship between a positive family history of hip fracture and risk of prevalent fracture at spine, hip or wrist. Specifically, the risk of prevalent hip fracture in those with a positive family history of hip fracture was not significantly increased, crude OR = 3.83 (0.44, 33.49). The wide confidence intervals of this estimate reflect the fact that the number of hip fractures in subjects and their first-degree relatives was small and the model is potentially unstable. The results relating family history of fracture to risk of fracture were also essentially unaltered if all reported osteoporotic fractures were included rather than including only those which had been validated.

The sensitivities, specificities, PPV and NPV of family fracture history for assessment of spinal and hip osteoporosis, and for any osteoporotic fracture at the wrist. hip and spine are shown in Table III.6. Site-specific analysis at the wrist showed that for a positive family history of wrist fracture the sensitivity to predict prevalent wrist fracture was 5%, the specificity 98.8%, the PPV 31.3% and the NPV 90.3%.

Table III.4 Odds ratio (95% CI) for fracture risk associated with a positive family history

Fracture site in study subjects	OR (Crude)	OR (Adjusted*)
Any fracture (n = 60 subjects)	2.02 (1.02, 3.78)	2.00 (1.04, 3.83)
Spine (n = 41 subjects)	1.20 (0.52, 2.74)	1.11 # (0.48, 2.59)
Appendicular (n = 22 subjects)	3.04 (1.21, 7.59)	2.70 (1.05, 6.92)
Wrist (n = 16 subjects)	2.91 (0.99, 8.50)	2.54 (0.85, 7.65)
Hip (n = 6 subjects)	3.17 (0.57, 17.45)	2.72 (0.48, 15.26)

* adjusted age, hip BMD, BMI

\# adjusted age, spine BMD, BMI

Table III.5 Odds ratio (95% CI) for fracture risk associated with a positive family history for osteoporotic fracture at wrist

	Prevalent vertebral fracture		Prevalent wrist fracture		Prevalent hip fracture	
Family fracture history	OR (Crude)	OR (Adjusted #)	OR (Crude)	OR (Adjusted *)	OR (Crude)	OR (Adjusted *)
Wrist	1.79 (0.77, 4.13)	1.87 (0.79, 4.41)	4.24 (1.44, 12.67)	4.74 (1.55, 14.51)	1.79 (0.21, 15.51)	1.81 (0.21, 15.95)

* adjusted age, hip BMD, BMI # adjusted age, spine BMD, BMI

Table III.6 **Sensitivity, Specificity, Positive Predictive Value (PPV), and Negative Predictive Value (NPV) of Family Fracture History for risk of osteoporosis and osteoporotic fracture**

	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Spinal Osteoporosis	15.2	91.0	21.2	87.1
Hip Osteoporosis	20.3	84.2	17.0	86.9
Any Osteoporotic Fracture	10.1	94.7	23.3	86.8
Spine Fracture	16.3	86.3	54.3	50.8
Hip Fracture	33.3	86.4	14.6	99.5
Wrist Fracture	31.3	86.5	3.6	55.3

III.5 Discussion

III.5.1 Twin study

The results presented in this chapter demonstrate that genetic factors contribute significantly to the variance in BMD at multiple skeletal sites in postmenopausal twins from the UK. The heritability estimates at spine, hip and forearm are similar in magnitude to those observed in studies of younger (Pocock et al, 1987) and older twins (Flicker et al, 1995), and also demonstrate a strong genetic correlation in the variance of total body BMD.

Compared to those obtained from studies in younger twins (Pocock et al, 1987), the rMZ estimates in these older twins are reduced at predominantly trabecular bone sites (lumbar spine: 0.74 vs 0.92, Ward's triangle: 0.71 vs 0.83). There was however little difference noted at cortical bone sites (femoral neck: 0.76 vs 0.73, distal radius: 0.71 vs 0.71). This suggests that unique environmental influences may become increasingly important with age at the trabecular sites rather than at the cortical bone sites. The finding that this site-specific reduction was not so apparent in the DZ twins may also suggest a stronger effect of the common environment within twin pairs on trabecular compared to cortical sites.

Limitations

The classical twin analysis makes 3 main assumptions: the trait means and variances are equal between the MZ and DZ pairs, equal common-environment between MZ and DZ pairs, and no evidence of gene interaction or epistasis. Slemenda et al (1991) have previously discussed the limitations of twin model and how results may be affected. In the data from this analysis, the mean values for the BMD measures were similar between the twin pairs (see Table III.1) and the TWINAN90 programme formally assesses the assumption of equal trait variances. The results did not suggest the presence of interaction or epistasis as the heritability estimates did not exceed 1.00 and the rMZ was not significantly greater than twice the rDZ value. Although the heritability estimates were essentially unaltered by adjustment for environmental confounders it is not possible to exclude the possibility of differences in the common environment between the zygosities.

The finding that the broad heritability estimates for BMD were similar to those in twin studies of different age ranges, does not imply that the genetic factors acting on bone mass are constant with time. Even if the genetic variance actually increased with age, the broad sense heritability could decrease with age if the variation in non-genetic factors increased more rapidly with age. Cross-sectional studies also cannot exclude the possibility of different genes being expressed during development, dependent on factors such as age, puberty or menopausal status.

III.5.2 Population study

In this cohort of women, history of fracture in a female first-degree relative was associated with a two-fold increased risk of osteoporotic fracture. This increase in risk appeared site specific, as a four-fold increased risk of wrist fracture was observed in subjects where a mother and/or sister had previously sustained fractures at this site. These data support a strong genetic component on factors associated with osteoporotic fracture, particularly at the wrist.

The results demonstrate that a history of osteoporotic fracture in a female first-degree relative is associated with a reduction in BMD at both spine and hip. The difference in BMD between the family history groups was equivalent to 0.4 SD at each skeletal site, and similar in magnitude to that observed in previous studies (Evans et al, 1988; Seeman et al, 1989; Tylavsky et al, 1989; Soroko et al, 1994; Seeman et al, 1994).

The data also suggest site specificity in genetic risk as a positive family history for appendicular fracture was associated with wrist and/or hip fractures but not with spinal fractures. It was not possible to analyse whether site specificity was also apparent for vertebral fractures as most vertebral fractures are asymptomatic and to accurately identify these in female first-degree relatives would have required radiological screening. Evidence for site specificity is also suggested by observations from other studies. Data from the SOF Study reported that in elderly women incident hip fracture risk was increased 2-fold in those with a positive maternal history of hip fracture. Other types of maternal fracture associated with falling did not, however, increase the risk of hip fracture (Cummings et al, 1995). In addition, further work from this group has recently

shown that parental history (both mother and father) of wrist fracture was associated with an increased risk of incident wrist fracture, whereas a family history of hip fracture was not associated with any increased risk (Fox et al, 1998). These increases in wrist and hip fracture risk were also independent of a subject's BMD at both the radius and hip respectively. A sibling history of wrist fracture was not associated with any increase in incident wrist fracture risk. Torgerson et al (1996) have also shown in a 2 year study of 1,857 perimenopausal women (age range 47-51 years) that the risk of any self-reported appendicular fracture was increased 3-fold in those with a maternal grandmother history of hip fracture. This increase in risk also appeared independent of a subject's BMD. Due to the younger age of this cohort no hip fractures were observed during the study period. In the EVOS Study, a parental history of hip fracture was not associated with an increased risk of prevalent vertebral deformity in women, although a modest association was observed between maternal history of hip fracture and vertebral deformity in men (Diaz et al, 1997). Other work has suggested that these deformities are more likely to be traumatic in aetiology and the importance of family history in this instance is therefore unclear (O'Neill et al, 1996; Silman et al, 1997).

The finding that the increase in fracture risk associated with a positive family history was unaltered after adjustment for BMD would suggest that common within family factors (both genetic and environmental) other than BMD may be contributing to the familial clustering of fracture risk. Bone structure and architecture have been shown to be under genetic control independent of BMD (Arden et al, 1996), and have also been demonstrated to be independent predictors of hip fracture in elderly populations (Hans et al, 1996; Faulkner et al, 1993). As family history data were based on appendicular fractures at the wrist and hip, this may indicate a possible familial component to the risk

of falling with the genetic effect mediated through factors such as muscle strength and proprioception (Arden and Spector, 1997)

Limitations

The study design used in the population-based analysis, being retrospective in design, is subject to several potential limitations. Prevalent fractures were determined by reported history and subsequently validated from patients' medical records. The validation process of these reported fractures has been described in detail previously (Arden et al, 1996). In total, 100% of reported hip fractures were validated and 66% of reported wrist fractures. The main reason that fractures could not be validated was that subjects had either died or moved away from the area and it was not possible to access their medical records. The proportion of reported fractures that were not subsequently validated did not, however, differ between the family history groups. Previous work has also shown that although elderly women tend to over-report fracture, self-report is accurate for fractures of the hip, wrist and humerus (Nevitt et al, 1992). The results were also essentially unaltered if all reported fractures rather than just validated fractures were analysed, although self-report of any fracture is less reliable (Nevitt et al, 1992). Only fractures relating to low trauma incident were studied in this analyses, although recent studies have also suggested that all traumatic fractures may be associated with reduced BMD (Sanders et al, 1998).

As with other studies utilising reported history of fracture in a relative (Soroko et al, 1994; Mallmin et al, 1994; Cummings et al, 1995; Torgerson et al, 1996; Diaz et al, 1997), the estimates of family history were based on recall only. It was therefore not

possible to determine whether differential recall bias in the reporting of family history was apparent between the subjects with and without prevalent fracture. Reporting of osteoporotic fracture in family members may have been biased by the subjects' awareness of their own fracture status, with fracture cases having better recall. This is particularly valid given that prevalent rather than incident fractures were assessed. In case-control studies it is difficult to measure the magnitude of recall bias in obtaining family history. Several studies have suggested that the ability of individuals to recall the presence of diseases in their own relatives may be quite limited (Schull and Cobb, 1969; Napier et al, 1972; Hastrup et al, 1985). If the rate of misclassification in reporting family information were the same between cases and controls (i.e. nondifferential) the estimated odds ratio would be underestimated. When, however, there is differential recall between cases and controls then the estimated odds ratio will differ. If cases over-reported positive family history then the estimated odds ratio would be falsely elevated, whereas if they under-reported positive family history compared to controls, then the estimated odds ratio would be lower than its true value.

Although differential recall bias cannot directly be excluded, no significant differences in either HRT or calcium/vitamin D use were observed between the family history groups. This suggests that at the time of the study's onset use of currently available treatments for osteoporosis was not influenced by knowledge of family history. At the time of completing the questionnaire all women were also unaware of their DXA BMD result, and only women with an age-matched Z-score of < -2.0 were subsequently notified as being a risk of osteoporosis.

Family history of disease should not, however, be considered as a simple attribute of a person. Family history depends on many factors such as the number of relatives, the biological relationship between the index case and their relatives, the age distribution of relatives, and the disease frequency within the general population. Osteoporotic fractures occur in later life and there is little evidence that this disease would therefore influence reproductive fitness or family size. Oestrogen exposure and increased parity have, however, been shown to be protective for osteoporosis in some studies (Alderman et al, 1986), suggesting that relatives from large sibships may have a lower risk for developing osteoporotic fracture. This would result in a downward bias in the frequency of positive family history among osteoporotic cases. Conversely, with large sibships and a common disease such as osteoporosis the probability of finding at least one affected relative of a case will be intrinsically greater than that of controls, even in the absence of any underlying difference in genetic risk. For example, the probability of an index case having a negative family history in a sibling is $(1 - k)^n$, where k is equal to the population risk and n is the number of siblings. For a disease with a population frequency of 5% to 10%, then it would obviously be quite possible to find one or more affected relatives by chance alone in large sibships. A deficiency of this study was that detailed information on the number of sisters in each family was not available. Information on fathers or brothers was also not recorded, and it was therefore not possible to confirm the reports that paternal history of fracture may be a more important risk factor than maternal history (Mallmin et al, 1994; Soroko et al, 1994; Fox et al, 1998).

III.6 Conclusions

The data presented in this chapter demonstrate a strong genetic component to BMD at multiple skeletal sites in postmenopausal twins, with heritability estimates ranging from 0.60 to 0.98. These estimates were independent of potential demographic and environmental confounders such as age, menopause duration, weight, and use of HRT. In unrelated women from the general population, a positive family history of fracture in a female first-degree relative is associated with reduced BMD at both the spine and hip, and with 2-fold increased risk of any osteoporotic fracture. There appears to be evidence for site specificity in this fracture risk as family history of wrist fracture was associated with a 4-fold increased risk of fracture at this site. The increases in fracture risk remained after adjustment for BMD, suggesting that other factors common within families (both genetic and environmental) may play an important role in the development of osteoporotic fractures.

CHAPTER IV

INFLUENCE OF VITAMIN D RECEPTOR GENOTYPE ON BONE MINERAL DENSITY IN PRE- AND POSTMENOPAUSAL WOMEN

IV.1 Summary

This chapter examines the relationship between polymorphisms at the VDR gene locus and bone mass in a study of 95 postmenopausal DZ twin pairs and in 1003 unrelated women from the general population. Twin subjects were genotyped for a synonymous *TaqI* polymorphism in the 3' region of the VDR gene and for a *FokI* polymorphism in the 5' coding region. The unrelated women were genotyped only for the 3' *TaqI* VDR gene polymorphism.

Within the DZ twins the *TaqI* polymorphism was associated with BMD at all skeletal sites measured, with a 7-10% difference in BMD between the homozygous genotypes. Weaker evidence for linkage between this polymorphism and BMD was also demonstrated at all sites apart from the lumbar spine. There was no evidence of linkage disequilibrium (LD) between the *TaqI* and *FokI* polymorphisms in the DZ twins, and there was no association between this latter marker and BMD.

In the women from the general population, there was no significant relationship between the *TaqI* polymorphism and BMD in both pre- and postmenopausal women. These findings were unaltered after adjustment for potential confounders such as age, weight, HRT use, smoking status and dietary calcium intake in both the twin subjects and the unrelated women.

IV.2 Introduction

At the onset of this thesis, Morrison et al (1994) had just reported on the relationship between VDR gene alleles and bone mass. In this study of predominantly premenopausal twins (64 MZ and 49 DZ pairs), BMD was measured using DXA at the lumbar spine and hip. VDR genotype was assigned using PCR and restriction endonuclease digestion with the enzymes *BsmI*, *ApaI* and *TaqI*. In this group, VDR genotype was specifically associated with BMD at the spine, and to a lesser extent at the hip, with the allele "B" (or "t") linked to low bone mass. Subjects of the VDR genotype "BB" were found to have a 10-15% reduction in BMD when compared to subjects with the "bb" genotype (equivalent to approximately 1 SD of the age-matched population distribution). A population based association study in 311 randomly selected unrelated women was also reported (Morrison et al, 1994), showing that women with the BB genotype were over represented in those subjects with BMD measurements 2 SD below the peak adulthood value (i.e. T score < -2). From these data it was subsequently estimated that the VDR locus was a major determinant of bone mass, explaining up to 75% of the genetic variation in BMD.

Because of the probability of type 1 errors, preliminary linkage and association studies require confirmation in different populations and enhance the candidacy of the locus. Having previously identified a genetic contribution to BMD and fracture risk in females from the UK population, the primary aim of this study was to examine the relationship between VDR genotype and BMD in these women in an attempt to replicate the original Australian findings.

IV.3 Methods

IV.3.1 Subjects

In total, 95 white, Caucasian female DZ twin pairs, concordant for postmenopausal status were studied. These subjects were aged 50-69 years of age and were recruited from the St. Thomas' Adult Twin Registry. All subjects were healthy and not known to be suffering from any disease affecting bone metabolism. BMD measurements at multiple skeletal sites were available for all subjects. VDR *TaqI and FokI* genotypes were obtained using standard techniques.

Unrelated women from the Chingford Study were also studied. BMD measurements were available in all subjects at the lumbar spine and hip. Genotype results for the VDR *TaqI* polymorphism were available on the majority of subjects.

IV.3.2 Statistical analyses

Two main analyses were performed:

1) Modified IBS sib-pair linkage analysis within the DZ pairs.

2) Association analysis within both the DZ twins and unrelated women.

IV.4 Results

IV.4.1 Twin analysis

Relationship between BMD and *TaqI* genotype

Full genotype and clinical information was available on all 95 DZ pairs. The genotype frequencies were similar to those previously reported in Caucasian populations (Morrison et al, 1994; Hustmyer et al, 1992) and were in HWE. Characteristics of the DZ twin group according to their VDR genotypes are shown in Table IV.1. Although small variations occurred between the different genotype groups, no significant differences were observed in age, BMI, age at menopause, smoking status or HRT use, although the heterozygous genotype "Tt" had a significantly higher weight than the homozygous genotypes ("TT", "tt").

IBS analysis showed that 55 pairs were concordant and 40 pairs discordant for *TaqI* VDR genotype, and no significant differences in baseline characteristics were observed between the groups. The correlation in BMD between concordant pairs was higher than the correlation between discordant pairs at all skeletal sites (Table IV.2), with the correlation values in the concordant pairs approaching those seen in MZ twins from this cohort. The difference between the concordant and discordant pairs was significant ($p < 0.05$) at the femoral neck, the total body and distal radius, although results were non-significant at the spine.

Table IV.1 Mean characteristics (SD) of 190 postmenopausal dizygotic twins according to VDR *TaqI* genotype

	TT (n = 75)	Tt (n = 78)	tt (n = 37)
Age (yrs)	58.9 (5.5)	58.3 (5.8)	59.2 (4.9)
Height (cm)	162.0 (5.6)	163.2 (6.5)	160.0 (6.3)
Weight (kg)	63.6 (9.1)	67.0 (12.8) #	63.8 (11.2)
BMI (kg/m^2)	24.3 (3.6)	25.2 (5.1)	25.0 (4.3)
Age at menopause (yrs)	48.9 (3.8)	48.8 (5.6)	48.9 (5.2)
Years since menopause (yrs)	10.0 (6.3)	9.5 (8.3)	10.3 (7.6)
No. ever use of HRT (%)	38 (51)	35 (45)	12 (32)
Median duration HRT use (months)	24 (6, 50)*	24 (9, 51)*	12 (3, 38)*
No. ever smoked (%)	41 (55)	40 (51)	18 (49)
Lumbar spine BMD (g/cm^2)	0.94 (0.15)	0.94 (0.17)	0.84 (0.16) ##
Femoral neck BMD (g/cm^2)	0.73 (0.11)	0.75 (0.12)	0.68 (0.10) ##
Ward's triangle BMD (g/cm^2)	0.58 (0.13)	0.58 (0.14)	0.49 (0.11) ##
Total hip BMD (g/cm^2)	0.85 (0.11)	0.87 (0.13)	0.79 (0.13) ##
Total body BMD (g/cm^2)	1.06 (0.10)	1.07 (0.15)	0.99 (0.11) ##
Distal radius BMD (g/cm^2)	0.43 (0.06)	0.43 (0.07)	0.39 (0.07)

* interquartile range

# $P < 0.05$ (vs TT/tt) ## $P < 0.05$ (vs TT/Tt)

Table IV.2 Intraclass correlation coefficients for skeletal site BMD in DZ twin pairs concordant and discordant for VDR *TaqI* genotype

	VDR Genotype	
Site	Concordant (n = 55)	Discordant (n = 40)
Lumbar spine	0.36	0.33
Femoral neck	0.52	0.31
Ward's triangle	0.39	0.26
Total hip	0.42	0.27
Total body	0.46	0.25
Distal radius	0.46	0.25

Analysis of all 190 subjects as independent subjects showed a significant association between VDR genotype and BMD at all skeletal sites other than the distal radius (Table IV.1, Figure IV.1). The "genotype group "tt" had lower BMD than the other 2 genotypes, with the "T" allele appearing to be dominant over the "t" allele. The difference between the homozygous genotypes was 7-10% across sites, equating to approximately 0.5-0.6 SD. These findings were similar when all 190 twins were analysed as individuals with adjustment for similarity or when 1 twin from each pair was randomly selected (n = 95 subjects). Results were also essentially unaltered after adjustment for age, weight, menopause duration and use of HRT. From the ANOVA analysis it appeared that the proportion of trait variance explained by VDR genotype was 4.5% at the lumbar spine, 4.5% at the femoral neck and 6.3% at the total body.

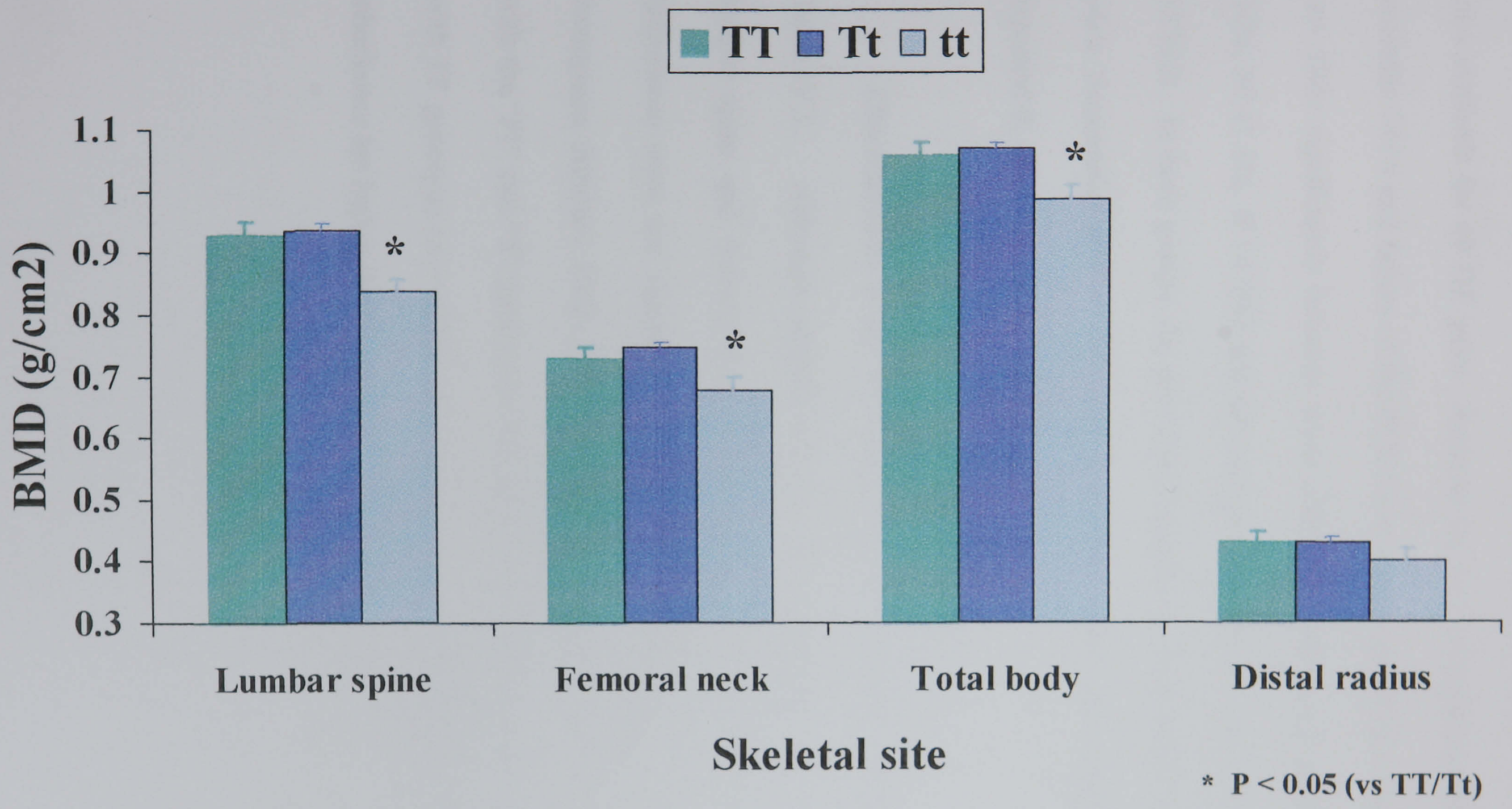


Figure IV.1 Mean BMD (± SE) at various skeletal sites in 190 DZ individuals according to *TaqI* VDR genotype

Relationship between BMD and *FokI* genotype

Genotype results were available on 159 postmenopausal individuals, with full data available for 69 DZ pairs. Reasons for lack of genotype data included absence of available DNA and failure of the PCR on at least 3 occasions. Genotype frequencies did not differ significantly between those observed in the total group of 159 subjects (FF 44%, Ff 41.5%, ff 14.5%) and the sub-group of 69 DZ pairs (FF 45.6%, Ff 40.6%, ff 13.8%). In both groups the genotype frequencies were again in HWE, with estimated allele frequencies of F = 0.65 and f = 0.35. These were similar to those previously reported in other Caucasian populations.

Characteristics of the DZ twins according to their *FokI* genotype are shown in Table IV.3. Although subjects with the "FF" genotype had lower BMD at both the lumbar spine and femoral neck when compared to the other two genotypes, these differences were not significant. Adjustment for potential confounders such as age, menopause duration, BMI, HRT use and smoking did not alter these findings. Subjects with the "Ff" and "ff" genotypes had significantly higher BMI when compared to those with FF genotype ($p = 0.003$ in both instances). This suggests a dominant pattern of inheritance for higher BMI associated with the "f" allele.

Table IV.3 Mean characteristics (SD) of 138 DZ twins according to *FokI* genotype

	FF (n = 63)	Ff (n = 56)	ff (n = 19)
Age (yrs)	59.1 (5.5)	58.7 (5.3)	59.5 (7.0)
BMI (kg/m^2)	23.7 (3.2)	25.7 (4.4) *	25.5 (4.1) *
Menopause age (yrs)	47.9 (5.5)	48.6 (3.7)	50.0 (3.7)
Menopause duration (yrs)	12.0 (7.2)	9.5 (5.6)	11.2 (8.8)
No. ever use of HRT (%)	25 (40)	18 (32)	4 (21)
No. ever smoked (%)	34 (54)	28 (50)	9 (47)
Lumbar spine BMD (g/cm^2)	0.91 (0.16)	0.93 (0.16)	0.92 (0.16)
Femoral neck BMD (g/cm^2)	0.71 (0.11)	0.74 (0.11)	0.73 (0.11)
Ward's triangle BMD (g/cm^2)	0.55 (0.14)	0.56 (0.13)	0.55 (0.13)
Total hip BMD (g/cm^2)	0.83 (0.12)	0.86 (0.12)	0.82 (0.12)
Total body BMD (g/cm^2)	1.03 (0.11)	1.06 (0.12)	1.05 (0.10)
Distal radius (g/cm^2)	0.42 (0.07)	0.44 (0.07)	0.41 (0.07)

* P = 0.003 (Ff vs FF, ff vs FF)

IBS analysis demonstrated 43 pairs concordant for *FokI* genotype and 26 pairs discordant. Both groups were comparable in their baseline characteristics. No relationship was observed in the DZ twins between their *FokI* IBS status and the intra-class correlation coefficients in BMD at the lumbar spine, femoral neck or any of the other skeletal sites measured using DXA (Table IV.4). Within-pair correlations were actually greater in twins discordant for *FokI* genotype rather than in those concordant, providing evidence against linkage of this polymorphism to BMD.

Table IV.4 Intra-class correlation coefficients for skeletal site BMD in 69 DZ pairs concordant and discordant for VDR *FokI* genotype

Skeletal site	*FokI* concordant (n=43 pairs)	*FokI* discordant (n=26 pairs)
Lumbar spine	0.23	0.56
Femoral neck	0.34	0.51
Total hip	0.37	0.43
Ward's triangle	0.26	0.44
Total body	0.28	0.56
Distal radius	0.21	0.61

Because an association between BMI and *FokI* genotype had been demonstrated, analysis was also performed looking at the intra-class correlations in BMI against their *FokI* IBS status. This showed that the intra-class correlation in BMI for concordant pairs was significantly higher than that seen in discordant pairs (0.52 vs 0.29, p = 0.01).

Relationship between the *FokI* and *TaqI* polymorphisms

TaqI and *FokI* genotype results were available in total on 156 DZ subjects. The distribution of subjects within the genotype groups is shown in table IV.5. The two loci appeared to segregate independently, with no evidence of linkage ($\chi^2 = 6.76$, P = 0.16). Formal statistical analysis also revealed no evidence for a significant interaction between the two loci acting on BMD (P > 0.10). The mean BMD values at the lumbar spine and femoral neck for each genotype group are shown in Figure IV.2.

Table IV.5 Distribution of subjects between the 2 VDR genotypes

	***FokI* Genotype**		
***TaqI* Genotype**	FF	Ff	ff
TT	31 (27)	24 (26)	8 (9)
Tt	31 (29)	23 (27)	11 (10)
Tt	7 (12)	17 (12)	4 (4)

Expected numbers are shown in parentheses

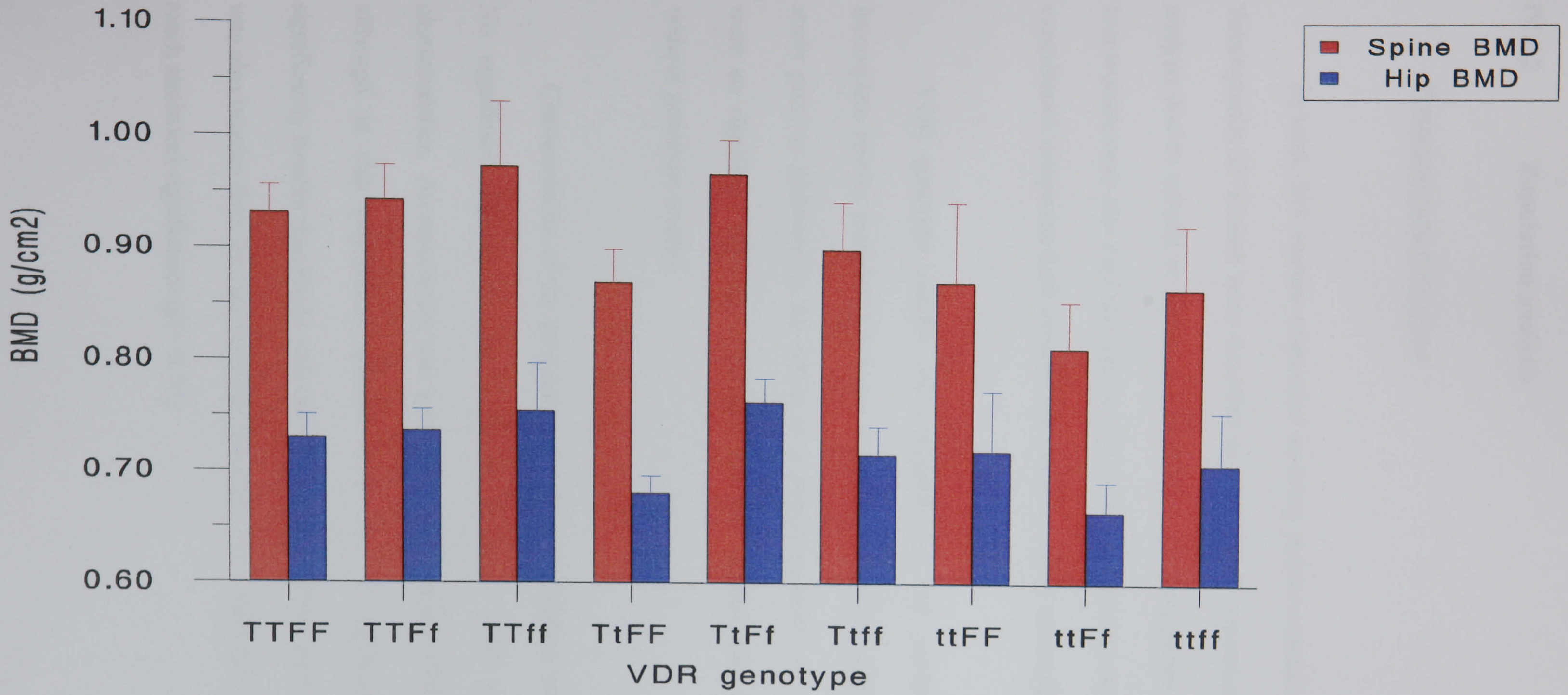


Figure IV.2 **Mean BMD (± SE) at spine and hip in DZ twins according to *TaqI* and *FokI* VDR genotypes**

IV.4.2 Population analysis

Premenopausal bone mass

In total, 213 women responded as being premenopausal at the initial interview. Subsequently 21 women were classified as peri- or post-menopausal and excluded from analysis due to current or previous use of HRT for menopausal symptoms. A further two women were also excluded due to previous hysterectomy, and although they had not experienced symptoms their menopausal status remained uncertain.

VDR genotype results were available on 141 women. The reasons for incomplete results included DNA not being available (i.e. subjects withdrawing from study prior to phlebotomy for DNA) or a persistent failure of the PCR assay. There were no significant differences between the 141 women with full results and the 49 without genotype results.

Characteristics of the premenopausal study population are shown in Table IV.6. No significant differences were observed between VDR genotype and baseline characteristics. An association was again observed between VDR genotype and weight, although in this population subjects with the "tt" homozygous genotype were significantly heavier than those with the "TT" genotype ($p = 0.03$). This genotype group was also heavier than the heterozygous genotype "Tt", although the results did not quite reach statistical significance ($p = 0.07$).

Table IV.6 Mean characteristics (SD) of 190 premenopausal women with stratification by *TaqI* VDR genotype in 141 subjects

		VDR Genotype		
Variable	Total group (n=190)	TT (n=53)	TT (n=65)	tt (n=23)
Age (yrs)	47.4 (2.1)	47.6 (2.2)	47.3 (2.0)	47.7 (2.5)
Height (cm)	162 (6)	162 (5)	161 (6)	163 (7)
Weight (kg)	66.0 (12.7)	64.7 (8.6) *	64.6 (12.8) #	70.5 (14.8)
BMI (kg/m^2)	25.0 (4.5)	24.5 (3.0)	24.9 (5.0)	26.5 (5.3)
Calcium intake (mg/day)	475 (223)	486 (218)	476 (225)	483 (227)
No. ever smokers (%)	76 (40%)	24 (45%)	28 (42%)	8 (35%)
Lumbar spine BMD (g/cm^2)	1.06 (0.13)	1.06 (0.12)	1.04 (0.13)	1.05 (0.11)
Femoral neck BMD (g/cm^2)	0.83 (0.11)	0.82 (0.10)	0.82 (0.11)	0.83 (0.09)

* P = 0.03 (vs tt)

\# P = 0.07 (vs tt)

Crude baseline BMD values at both the spine and hip did not differ significantly between the *TaqI* genotypes. These findings were unaltered after adjustment for the observed difference in body weight. Adjustment for other potential confounders such as age, smoking status and dietary calcium intake also did not alter these results.

Postmenopausal bone mass

In total, 725 women reported being postmenopausal at the initial interview. Characteristics of these women are shown in Table IV.7. *TaqI* genotype results were available on 432 of these (60%). Reasons for lack of genotype results are similar to those previously reported for those of the premenopausal women.

No significant differences in crude BMD at either the lumbar spine or hip were observed between the *TaqI* genotypes. VDR genotype did, however, appear to be again associated with body weight, although here the heterozygote genotype "Tt" was significantly heavier than the homozygote genotype TT ($p < 0.05$). Adjustment for this observed difference in weight did not, however, alter the negative relationship between VDR genotype and BMD. Results were also unaffected by adjustment for age, menopause duration, dietary calcium intake, smoking status and ever use of HRT.

Table IV.7 Mean characteristics (SD) of 725 postmenopausal women with stratification by *TaqI* VDR genotype in 432 subjects

		VDR Genotype		
Variable	Total group (n=725)	TT (n=153)	TT (n=211)	tt (n=68)
Age (yrs)	56.7 (5.1)	56.2 (4.8)	56.5 (5.1)	56.4 (5.3)
Height (cm)	161 (6)	161 (6)	162 (6)	161 (6)
Weight (kg)	67.3 (11.7)	65.5 (9.8)	68.2 (11.5) *	65.7 (11.8)
BMI (kg/m^2)	25.8 (4.3)	25.2 (3.5)	26.1 (4.1)	25.4 (4.1)
Menopause duration (yr)	8.5 (5.9)	7.6 (5.9)	8.6 (6.2)	7.8 (5.5)
Calcium intake (mg/day)	481 (240)	485 (260)	489 (233)	472 (209)
No. ever smokers (%)	338 (47%)	77 (50%)	87 (41%)	29 (43%)
No. ever use HRT (%)	187 (26%)	28 (18%)	51 (24%)	15 (22%)
Lumbar spine BMD (g/cm^2)	0.94 (0.16)	0.92 (0.15)	0.95 (0.13)	0.93 (0.17)
Femoral neck BMD (g/cm^2)	0.74 (0.12)	0.73 (0.12)	0.73 (0.11)	0.74 (0.11)

* $P < 0.05$ (vs TT)

IV.5 Discussion

Relationship between *TaqI* VDR genotype and BMD

The data from the twin analysis demonstrated evidence for association and supportive evidence of linkage between a *TaqI* polymorphism in the 3' region of the VDR gene and bone mass at several skeletal sites of differing bone composition and skeletal loading. The genotype "tt" was specifically associated with a 7-10% reduction in BMD (equivalent to 0.5 SD) when compared to the other genotypes, and results were unaffected after adjustment for potential environmental confounders. These results were similar in both the twin-based analysis and in the association-based analysis that ignored the effects of pairing, although at the lumbar spine a significant VDR *TaqI* genotype-BMD relationship was only observed in the association analysis. The population analysis failed, however, to show a similar relationship between *TaqI* VDR genotype and BMD at either the spine or femoral neck. There was no association in either pre- or postmenopausal subjects, and the results were again unaffected by potential environmental confounders.

These results demonstrate that the effect of the VDR locus on BMD is several orders of magnitude lower than the initial estimate of Morrison et al (1994), perhaps explaining less than 5% of trait variance with only a 0.5 SD difference in BMD between the homozygous genotypes. The validity of the Australian twin data figures is now, however, questioned due to partial retraction of their original findings (Morrison et al, 1997) as some of the genotyping contained errors. Reanalysis of this work is still

reported to demonstrate a borderline effect between VDR genotype and BMD in the twin subjects, although no precise estimate has to date been published.

To date, only one other study (Hustmyer et al, 1994) has been published where the relationship between BMD and VDR genotype has been specifically examined in twins (86 MZ and 39 DZ predominantly premenopausal pairs). In this study, BMD was measured at several sites (lumbar spine, hip, forearm), and VDR genotype was determined with individual digestion of genomic DNA with 3 restriction endonucleases (*TaqI*, *BsmI*, *ApaI*), and Southern blot hybridisation with a radiolabelled 1.4 kb VDR cDNA. The study was originally only powered to detect a 1 SD difference in BMD between the homozygous genotypes, as described by Morrison et al (1994), although larger numbers would be required to detect smaller differences similar to those observed in this thesis. Only an association-based analysis was performed on all twins independent of their zygosities and pairings. Results were subsequently stratified for menopausal status and HRT use. Overall, there was no significant association identified between any of the three RFLPs and BMD. In addition, in DZ twins discordant for genotype no allele was specifically associated with either lower or higher BMD values.

Following the report by Morrison et al (1994), a large number of population-based studies have subsequently been published examining the relationship between the 3'-VDR polymorphisms and BMD. Both positive and negative associations have been observed, and the reasons for the conflicting results are again not immediately apparent. Studies up to 1995 were summarised by Peacock (1996), with more recent publications detailed by both Cooper and Umbach (1996) and Gong et al (1999). Cooper and Umbach (1996) used a meta-analytic approach to assess quantitatively the association between VDR and BMD from 16 of these published studies up to July 1996. There was strong evidence of study-to-study heterogeneity in effect size at both the spine and hip.

They reported that there appeared to be a modest effect of VDR genotype on BMD, with a reduction of about 2% in the "BB" compared to "bb" genotype groups. The magnitude of any effect seemed most marked in younger subjects and appeared to reduce with age. Interestingly, removal of the Australian twin data (Morrison et al. 1994) reduced these effect size estimates that were then no longer significant. Gong et al (1999) identified 75 articles and abstracts that had been published up to the end of December 1996. In total, 34.3% of these studies reported a significant association between VDR genotype and spine BMD, with 43.1% reporting an association with femoral neck BMD. Despite obvious limitations of publication bias, they claim that these results are greater than one would predict by chance and support the finding of a significant relationship between VDR genotype and BMD. In general the positive studies were again more commonly seen in studies of younger subjects.

Power calculations for the sample sizes required for many of the published studies would have been based on the prediction that the VDR gene explained up to 75% of the genetic variance acting on BMD (Morrison et al, 1994). Work from this thesis, and from the meta-analysis of Cooper and Umbach (1996) would suggest, however, that the effect attributable to the VDR locus is much smaller and that many of the early studies were therefore too small and probably under-powered to detect such modest genetic effects. Other studies have speculated that dietary intakes of calcium and vitamin D may also affect the relationship between VDR genotype and BMD (Dawson-Hughes et al, 1995; Krall et al, 1995; Graafmans et al, 1997; Kiel et al, 1997; Ferrari et al, 1998), although this has not been observed in all studies (Garnero et al, 1996b). Vandevyver et al (1997) have also noted an effect after stratification for BMI, with a

VDR genotype effect at the femoral neck only observed in elderly non-obese subjects (BMI < 30 kg/m^2).

One possibility for the conflicting results from the association studies could be due to LD between the *TaqI*/*BsmI* polymorphisms and either other sequence changes in the VDR gene or with another locus mapping near to this chromosomal region 12q12-14. This concept is supported by the findings of 3 studies demonstrating association between VDR genotype and bone mass, but where the association was with the alternate allele (Salamone et al, 1996; Houston et al, 1996; Uitterlinden et al, 1996). To date, no study has examined the association between the 3' poly-A sequence and BMD, despite the knowledge of strong LD between this and the *BsmI* VDR polymorphism. Potential candidate genes mapping to chromosome 12 that could have effects on bone mass, either directly or indirectly, include the insulin-like growth factor type 1 (IGF-1), type II collagen α1 (COL2A1), and nitric oxide synthase.

Relationship between *FokI* genotype and BMD

Analysis of a *FokI* polymorphism in the 5' region of the VDR gene failed to show any evidence of either linkage or association between the *FokI* polymorphism and bone mass in the postmenopausal twins. To date, only five published studies have examined the relationship between the *FokI* polymorphism and BMD using an association based analysis in unrelated individuals (Gross et al, 1996; Arai et al, 1997; Harris et al, 1997; Eccleshall et al, 1998; Ames et al, 1999). These studies and their results are summarised in Table IV.8

Table IV.8 **Summary of association studies examining the relationship between *FokI* VDR genotype and BMD**

					Δ BMD (genotype ff vs FF)			
Study	No. subjects	Ethnic group	Menopausal status	Age range (yrs)	LS	FN	WB	R
Gross (1996)	100	Caucasian	Post-	59-82	↓ 13%	NS	-	NS
Arai (1997)	110	Oriental	Pre-	24-45	↓ 12%	-	-	-
Harris (1997)	72	Black	Pre-	20-40	NS	NS	NS	-
Harris (1997)	82	Caucasian	Pre-	20-40	NS	↓ 12%	↓ 4%	-
Eccleshall (1998)	174	Caucasian	Pre-	31-56	NS	NS	NS	NS
Ames (1999)	72	Mixed	Prepubertal	7-12	-	-	↓ 8 %	-

LS: lumbar spine FN: femoral neck WB: whole body R: radius

NS: non-significant

No consistent trend in the results is apparent across studies, suggesting that any effect attributable to this polymorphism is modest and variable. Ames et al (1999) observed a relationship between calcium absorption and *FokI* genotype, with subjects of the "FF" genotype having a mean calcium absorption 41.5% greater than those of the "ff" genotype and 17% greater than the "Ff" heterozygotes. This suggested that the effect of this polymorphism on BMD could also be influenced by dietary calcium intake.

As the *FokI* polymorphism maps to the 3' region of the VDR gene, it was reassuring to note there was no observed evidence of LD with the *TaqI* RFLP. There was therefore no need to look for evidence of interaction between the two polymorphisms.

Relationship between VDR genotypes and body weight

The findings of an association between the *TaqI* polymorphism and weight in both the twin and population samples, and between the *FokI* polymorphism and BMI in the twins are of interest. Harris et al (1997) also demonstrated that white women with the "ff" genotype were significantly heavier (on average 8kg) than women with the other two genotypes. A similar trend was also observed by Eccleshall et al (1998), with women of the "Ff" and "ff" genotypes tending to be non-significantly heavier than those of the "FF" genotype (4kg and 2 kg respectively). Other published studies have not, however, consistently observed this observation.

Interestingly, the mouse VDR knockout was shown to have evidence of impaired growth and failure to thrive in the post-weaning stage, suggesting an effect of this locus

on growth (Yoshiizawa et al, 1997). The VDR locus has also been shown to influence weight in infancy, suggesting a role in the control of body size (Keen et al, 1997). It is not clear from this data whether the VDR genotype association with weight relates to either fat or lean mass, or whether the influence is independent of any known association with BMD. Model-fitting analysis of twin data in 57 MZ and 55 DZ pairs has demonstrated strong genetic regulation of fat mass, lean mass and BMD, although associations between these traits was largely mediated via environmental factors (Nguyen et al, 1998). Multivariate analysis in a larger sample of twin pairs would allow the effect attributable to the VDR locus to be quantified and determined (Martin et al, 1997).

Limitations

The twin-based modified sib-pair linkage analysis had modest power due to the small sample size of 95 pairs and the fact that only an IBS classification was used. The absence of parental information on the twin subjects also greatly reduces the informativeness of the IBS classification (Bishop and Williamson, 1990), as does the low heterozygosity indices for the biallelic genetic markers: *TaqI* (H = 0.48) and *FokI* (H = 0.46). The collection and use of parental genotype information and/or the use of more informative markers (i.e. microsatellite polymorphisms rather than a biallelic RFLP) would have increased the informativeness of the linkage analysis.

The association-based analysis in the twins utilised only data from one member of each pair, to reduce the bias from using correlated samples. By effectively halving the sample size, this approach does not maximise the potential of the data and increases the

risk of a type II error. A different approach would have been to utilise the generalised estimating equation (Zeger and Liang, 1986). This method takes into account the dependence of measurements within twin pairs in estimating the significance of the differences and utilises all the information. Association-based approaches also appear to have increased power compared to linkage to detect loci having modest effects on disease risk (Risch and Merikangas, 1996).

The population sample had adequate power (80%) to detect an effect on BMD attributable to the VDR locus of 0.5 SD. Compared to the twins, who were recruited nationally, the unrelated women were all from a single geographical region of NE London. Although it is possible that a localised environmental agent may be masking or overriding the effect of the VDR gene on BMD in this group, no significant differences in common environmental factors were observed. The lack of calcium intake data in the twins may, however, have been important and should be assessed. It is possible that the VDR gene effect on BMD is only seen in twins, although the negative findings of Hustmyer et al (1994) would not support this hypothesis.

In the twin analysis, discrepant results were observed between the twin-based and individual-based analyses at the lumbar spine. The lumbar spine BMD values may have been influenced by degenerative disease (Masud et al, 1993), and this could have specifically affected the twin-based analysis as there is an independent genetic component acting on this trait (Sambrook et al, 1999). Unfortunately spinal radiographs were not available on the twin subjects to evaluate the presence and extent of degenerative change.

A final potential limitation was that this study examined each VDR polymorphism individually. The *TaqI* polymorphism was chosen because of documented strong LD between it and the *BsmI* site (Morrison et al, 1994), presence of an invariant (control) site for restriction endonuclease digestion, and also because of cost. Results from Uitterlinden et al (1996) suggest that haplotype analysis may be more informative. and this is supported by recent studies in other fields (Nickerson et al, 1998; Keavney et al. 1998). Lack of parental information would mean that it would not be possible to unambiguously assign haplotypes, so these would need to be estimated using derived population frequencies. Haploptypic analysis of quantitative traits such as BMD would require larger sample sizes than available in this study, and raises concerns about multiple testing issues (Altshuler et al, 1998).

IV.6 Conclusions

Within postmenopausal DZ twins a *TaqI* polymorphism in the 3' region of the VDR gene appeared associated with BMD across spine, hip and total body sites. with the "t" allele specifically associated with low bone mass. Weaker evidence for linkage between the VDR locus and BMD at all sites other than the lumbar spine was also demonstrated. Analysis of the 5' *FokI* polymorphism in the DZ twins failed to find any association or linkage with BMD at any of the skeletal sites. A population-based analysis in pre- and postmenopausal women failed to replicate these findings, with no significant association between the *TaqI* genotype and BMD at either the spine or hip. The reason for these conflicting results was not clear, although environmental differences between the groups could have contributed and further research should explore the role of potential gene-gene and gene-environment interactions acting on BMD.

There also appeared to be a consistent relationship between VDR genotype and aspects of body weight in both study groups. The *TaqI* genotype was significantly associated with body weight in both the twin and population based analyses, whilst in the twins the *FokI* genotype was significantly associated with body mass index, with additional supportive evidence for linkage. It remains to be determined whether this association with weight relates to body size or whether there is a relationship between VDR genotype and either fat or lean mass.

CHAPTER V

INFLUENCE OF VITAMIN D RECEPTOR GENOTYPE ON EARLY-POSTMENOPAUSAL BONE LOSS AND OSTEOPOROTIC FRACTURE

V.1 Summary

The previous chapter has demonstrated an association between a *TaqI* polymorphism in the 3' region of the VDR gene with BMD in postmenopausal DZ twins. Similar findings were not, however, observed in unrelated women from the UK general population. As earlier work in this thesis had suggested a familial risk to fracture that was independent of BMD, this chapter has examined whether the VDR *TaqI* genotype was associated with osteoporotic fracture and with rates of menopausal bone loss.

Longitudinal measurements of BMD at spine and hip were available on women who were within 5 years of a natural menopause. Significant bone loss occurred at these skeletal sites over an average of 3-4 years, although there was no association between the *TaqI* VDR genotypes and this bone loss. Prevalent osteoporotic fractures were assessed at spine, hip and wrist in all subjects. Overall, these was no increase in total fracture risk between the *TaqI* VDR genotypes, although the small number of fractures at each site precluded site-specific analysis.

These data therefore exclude the VDR gene having a major influence on determining rates of change in BMD in postmenopausal women or on increasing risk of osteoporotic fracture. Future work should be targeted to identify other genes influencing risk of osteoporosis, and to examine for possible gene-gene interactions.

V.2 Introduction

Epidemiological studies have demonstrated that BMD in later life is a strong predictor of subsequent fracture (Cummings et al, 1990; Cummings et al, 1993). When studying individuals with and without fractures there is still considerable misclassification and overlap of low and normal bone density results, with an average of only 0.5 standard deviations between cases and controls. In addition, and more importantly, BMD still fails to explain a considerable portion of the variance in fracture risk (Ott 1993). Data from Chapter III of this thesis and from other epidemiological studies (Cummings et al, 1995; Fox et al, 1998) have demonstrated a familial risk to fracture that is independent of BMD. This suggests that genetic factors may also be acting on other intermediate traits that may ultimately increase the risk of fracture.

In this chapter, the relationships between *TaqI* VDR genotype and both longitudinal measures of menopausal bone loss, and osteoporotic-related fractures has been examined in a population-based analysis.

V.3 Methods

Data was available on all 1,003 women from the Chingford Study. For the study of early postmenopausal bone loss, analysis was performed on a subset of women who were within 5 years of a natural menopause and where annual measurements of BMD were available for longitudinal assessment of bone loss. DNA and VDR *TaqI* genotype results were available on the majority of subjects.

Standard statistical analytical techniques were utilised to examine the associations between VDR genotype and the phenotypic traits of interest. Differences in demographic variables between VDR genotypes were initially compared using ANOVA for quantitative traits and chi-squared test for qualitative traits. Adjustments for potential environmental and demographic confounders were made using ANCOVA. Conditional logistic regression analysis was used to estimate the odds ratio and 95% test based confidence intervals for fracture risk associated with the individual VDR genotypes under dominant, recessive and co-dominant inheritance models. Adjustment for other potential confounding variables associated with fracture risk was also performed using conditional logistic regression.

V.4 Results

Early postmenopausal bone loss

Full results (3 or more scans and *TaqI* genotype) were available on 125 women. For this total group the mean (95% confidence interval) rate of change from the lumbar spine was -1.1 %/yr (-1.32, -0.88) and from the femoral neck was -0.76 %/yr (-1.08, -0.45). These rates of change were both statistically significantly different from zero.

No significant differences were observed in the baseline characteristics between the 3 VDR genotypes (Table V.1). Overall, rates of bone loss at the spine and hip did not differ significantly between the VDR genotypes, and these findings were unaltered

after adjustment for potential confounders (i.e. age, menopause duration. weight. smoking status, initial BMD) or after stratification for dietary calcium intake.

Table V.1 **Mean characteristics (SD) of 125 early postmenopausal women**

	VDR Genotype		
	TT (n=45)	Tt (n=61)	tt (n=19)
Age (yrs)	52.8 (3.7)	52.5 (3.5)	52.6 (3.3)
Age at menopause (yrs)	50.5 (3.7)	50.2 (3.4)	50.4 (2.8)
Menopause duration (yrs)	2.2 (1.3)	2.3 (1.4)	2.2 (1.4)
Height (cm)	161 (6)	161 (18)	162 (5)
Weight (kg)	66.0 (11.0)	67.0 (13.3)	66.6 (14.1)
BMI (kg/m^2)	25.3 (4.2)	25.6 (4.1)	25.3 (4.7)
Change in spine BMD (%/yr)*	-1.31 (0.14)	-1.09 (0.17)	-1.01 (0.30)
Change in hip BMD (%/yr)*	-0.48 (0.36)	-1.00 (0.20)	-1.00 (0.46)

* Mean (SE)

Osteoporotic fractures

Full genotype results and validated fracture data were available on 595 women. The distribution of VDR genotypes did not differ significantly between subjects with and without prevalent osteoporotic fractures, or when analysis was restricted specifically to vertebral or wrist fracture (Table V.2). The "t" allele was not associated with an increased risk of fracture under recessive or dominant inheritance patterns, and these findings were not altered after adjustment for potential confounders such as age, BMI, and hip BMD (Tables V.3 and V.4).

Table V.2 Distribution of subjects (%) according to their VDR genotype and fracture status

VDR Genotype	No fracture (n=542)	Any fracture (n=53)	Vertebral fracture (n=29)	Wrist fracture (n=9)
TT	199 (36.7)	19 (35.8)	11 (37.9)	4 (44.4)
Tt	257 (47.4)	27 (50.9)	15 (51.7)	4 (44.4)
tt	86 (15.9)	7 (13.3)	3 (10.4)	1 (11.2)

Table V.3 **Odds ratios (95% CI) for fracture associated with the tt genotype vs the TT/Tt genotypes**

Fracture status	Crude OR
Any fracture	0.67 (0.31, 1.45)
Vertebral fracture	0.62 (0.18, 2.08)
Wrist fracture	1.09 (0.24, 5.06)

Table V.4 **Odds ratios (95% CI) for fracture associated with the tt/Tt genotypes vs the TT genotype**

	Crude OR
Any fracture	1.07 (0.64, 1.79)
Vertebral fracture	0.93 (0.43, 2.00)
Wrist fracture	0.57 (0.18, 1.78)

V.5 Discussion

Relationship between *TaqI* VDR genotype and postmenopausal bone loss

These data did not demonstrate any significant association between *TaqI* VDR genotype and rates of change in BMD at either spine or hip. Adjustment for potential confounders also did not alter these negative findings. Rates of bone loss at both skeletal sites were normally distributed and it was not possible to identify a sub-group of women who would be classified as "fast bone losers". Although rates of change in BMD were modest they were significantly different from zero.

These results contrast with the cross-sectional population study by Morrison et al (1994) that suggested an association between VDR genotype and rates of change in BMD. Extrapolation of these data demonstrated similar BMD values for the 3 *BsmI* genotypes at the onset of the menopause, with the regression slope of BMD against time appearing steeper for the "BB" genotype group compared to either the "bb" or "Bb" groups. Subsequent population based studies with longitudinal DXA data have, however, not supported these observations (Kroger et al, 1995; Berg et al, 1996; Garnero et al, 1996b; Jorgensen et al, 1996; Hansen et al, 1998). Several studies have, however, observed significant associations between VDR genotype and markers of bone turnover (Kruger et al, 1995; Howard et al, 1995; Tokita et al, 1996). These findings are, however, not consistent between studies (Garnero et al, 1996b; Tsai et al, 1996; McClure et al, 1997; Kung et al, 1998).

Several studies have examined the relationship between VDR genotype and BMD response to calcium and/or vitamin D in therapeutic trials. At the hip, rates of change correlated with VDR genotype in a study of 229 postmenopausal women receiving calcium supplements (Krall et al, 1996), and in a study of 81 postmenopausal women receiving 400 IU vitamin D_3 (Graafmans et al, 1997). In these studies, the genotype "BB" appeared more responsive to therapy with either calcium or vitamin D. No data on lumbar spine BMD were available in these studies, probably reflecting the subjects' age and the fact that degenerative spinal disease would have affected longitudinal BMD measurements. Two smaller studies have, however, observed an association between rates of change in lumbar spine BMD and VDR genotype in subjects receiving calcium supplements (Ferrari et al, 1995) and 1α-hydroxyvitamin D_3 (Matsuyama et al; 1995). There was no consistent genotype association in these two studies, with the study by Ferrari et al (1995) observing a correlation between change in spine BMD and dietary calcium intake only in the heterozygous genotype "Bb", whilst the study by Matsuyama et al (1995) noted an increased therapeutic response in the haplotype "bbaaTT" compared to the others. These findings suggest a gene-environment interaction influencing rates of change in BMD. Adjustment for dietary calcium intake did not influence the results presented in this chapter. Two of the population-based studies quoted earlier did stratify their analyses according to either calcium intake (Garnero et al, 1996b) or calcium intake and serum 25-hydroxyvitamin D_3 (Hansen et al, 1998), although this did not alter their negative findings. The mean dietary intake of calcium was between 800-900 mg/day in these studies compared to pre-treatment intakes of less than 650 mg/day in the studies by Ferrari et al (1995) and Krall et al (1995). This suggests that the VDR genotype relationship with bone loss may only be apparent in subjects with low dietary intakes of calcium.

The number of subjects available for analysis limits the findings in this Chapter. Despite this, the study had approximately 70% power to detect a 1% difference in rates of change in BMD between the homozygous genotypes. Although larger studies would be required to have more power, the present study precludes the VDR gene locus as having a major and probably clinically important influence on rates of change in BMD. The modest rates of bone loss observed in this study, the duration of follow-up, and the precision error associated with the DXA BMD measurements also precluded identifying small differences between the genotypes. Although forearm BMD measurements using SPA have a low precision error, this predominately cortical bone site does not respond as greatly to oestrogen deficiency as trabecular bone at the spine. Studies of longer duration will be required to identify small differences in rates of change between the genotypes, although again whether these would be clinically important is uncertain.

Relationship between *TaqI* genotype and osteoporotic fracture

Overall, no relationship was observed between VDR genotype and history of prevalent fracture. As earlier work in this thesis has suggested site specificity to fracture risk, analysis was also performed examining the genotype-association with both prevalent vertebral and wrist fractures. The number of subjects with osteoporotic fractures at these two skeletal sites was small, and results showed no association. It was not possible to demonstrate an increased fracture risk for the "T" allele under either recessive or dominant inheritance models.

To date, only one small study has identified an association between VDR genotype and vertebral fracture in 25 Japanese patients (Yangi et al, 1996). Increased fracture risk was associated with the "B" allele, with a co-dominant or additive inheritance model. Beavan et al (1996) and Young et al (1996) both reported that hip fracture prevalence correlated with population frequency of the "B" or "t" allele, and this could explain in part the ethnic differences observed in fracture rates. They note that the "B" ("t") allele is rare in Asian subjects, although Yangi et al (1996) reported a 14-fold increased fracture risk associated with the "BB" genotype. The low population frequency means it can only explain a small proportion of Japanese osteoporotic fracture cases.

A large number of other studies have also examined the genotype distributions in subjects with and without vertebral fracture, finding no significant association (Melhus et al, 1994; Lim et al, 1995; Looney et al, 1995; Berg et al, 1996; Houston et al, 1996; Francis et al, 1997, Vandevyver et al, 1997; Ensrud et al, 1999). Nguyen et al (1994) estimated that studies of greater than 300 subjects may be required to detect loci having modest effects on fracture (i.e. relative increase in risk of 1.4). Individually these studies may have been under-powered, although pooling of results provides a total sample size of more than 1000 subjects. Accepting that there may be differences in study design and patient entry criteria, overall this analysis shows no difference in VDR genotype frequencies between the control subjects and vertebral fracture cases (Figure V.1). These results would therefore suggest that the VDR locus does not influence the risk of vertebral fracture, although further studies will be required to examine this relationship in more detail at the appendicular sites of hip and wrist.

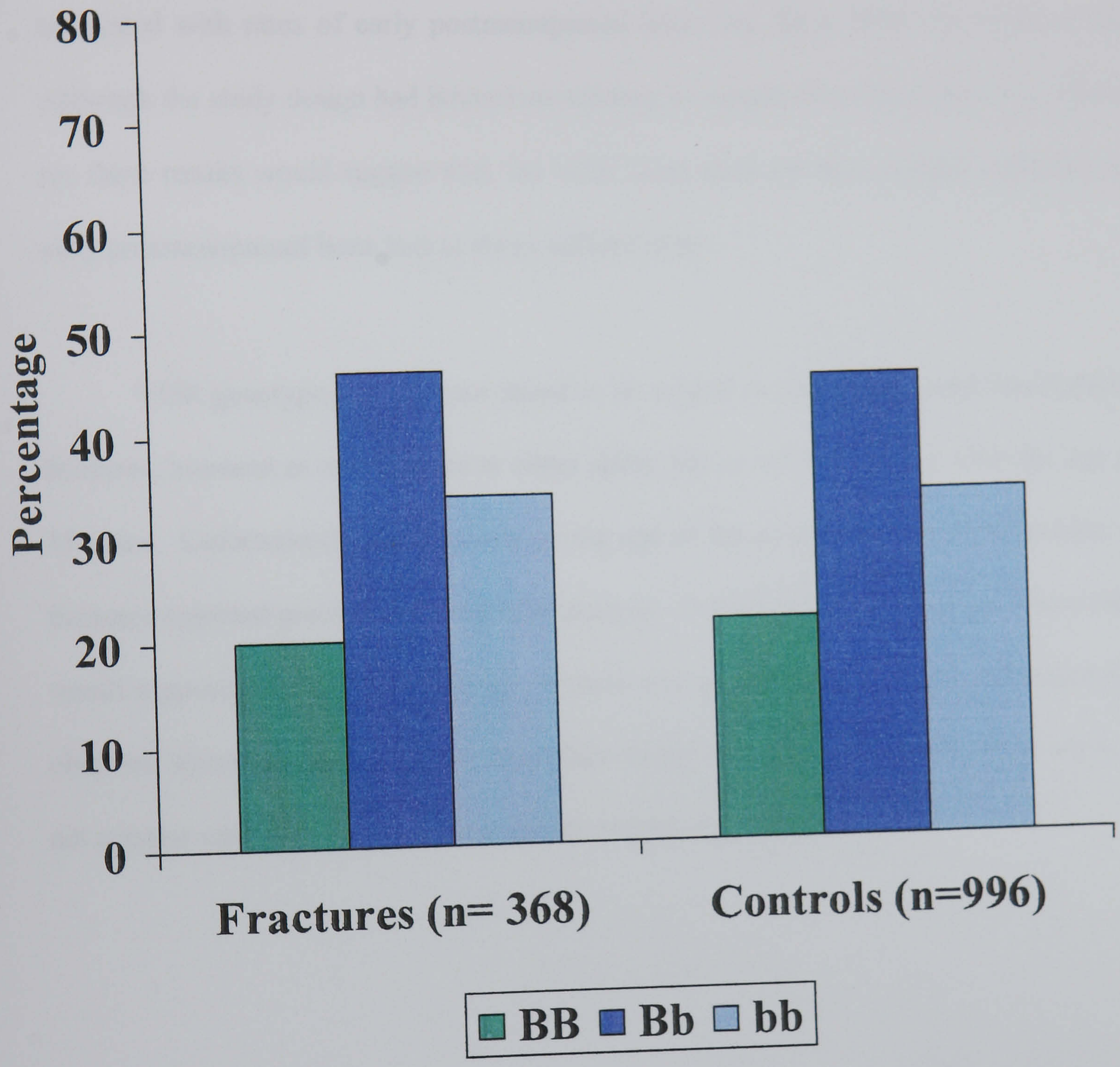


Figure V.1 **Pooled results of VDR *BsmI* genotype distributions between vertebral fracture cases and controls**

V.6 Conclusions

In a population-based analysis, VDR *TaqI* genotype was found to not be associated with rates of early postmenopausal bone loss from either the spine or hip. Although the study design had limitations relating to sample size and duration of follow-up, these results would suggest that the VDR locus does not have a major influence on early postmenopausal bone loss at these skeletal sites.

VDR genotype was also not found to be related to risk of prevalent osteoporotic fractures, assessed as any fracture at either spine, hip or wrist occurring after the age of 35 years. Unfortunately the relatively young age of the cohort and the small number of fractures reported precluded site-specific analysis. Comparison with other published data would support these negative findings, as there was no consistent increase in fracture risk observed across studies. Overall these data would also suggest that the VDR locus is not a major candidate gene for determining osteoporotic fracture.

CHAPTER VI

CANDIDATE GENE ANALYSIS IN OSTEOPOROSIS: POPULATION BASED ASSOCIATION STUDIES OF THE OESTROGEN RECEPTOR, TYPE I COLLAGEN α1 AND INTERLEUKIN 1 RECEPTOR ANTAGONIST GENES

VI.1 Summary

It would appear that the VDR gene locus contributes only modestly to the population variance in BMD, and this chapter has therefore examined the association between three additional candidate genes (oestrogen receptor, type I collagen α1, and interleukin-1 receptor antagonist) and osteoporosis risk traits. Selection of these candidate genes was based on knowledge of bone cell biology and presumed disease pathophysiology. In addition, evidence for interaction between these loci and the VDR gene has also been explored.

Overall, there was no association between polymorphism at the oestrogen receptor locus and osteoporosis related traits of BMD or early menopausal bone loss. At the IL-1RN locus, however, a polymorphic VNTR was associated with differential rates of bone loss at the lumbar spine but not at the hip. No relationship was observed between the IL-1RN VNTR and baseline BMD at these two skeletal sites. At the COL1A1 locus a significant association between BMD and fracture risk was observed. Carriage of at least one copy of the COL1A1 "s" allele was associated with reductions of 0.25 SD in BMD at both spine and hip, and a 2-3 fold increased risk of any osteoporotic fracture. There was also evidence of interaction between the VDR locus and the COLIA1, with fracture risk markedly increased in subjects who had at least one copy of the "s" allele and who were heterozygous for the *TaqI* polymorphism.

These data therefore suggest that osteoporosis is a complex disease as several genes influence disease risk with evidence of gene-gene interactions.

VI.2 Oestrogen receptor

VI.2.1 Introduction

Oestrogen appears to play an important role in maintaining bone mass in both adult men and women by suppressing bone remodelling and maintaining a balance between osteoblastic and osteoclastic activity. The skeletal action of oestrogen appears to be mediated through its intracellular receptor (ER), which has been shown to be expressed in bone (Eriksen et al, 1988). Studies of the ERα-knockout mouse (Lubahn et al, 1993) and from an ERα human gene mutation (Smith et al, 1994) provide direct evidence for the ER having effects on the regulation of bone mass.

Although gross germline structural mutations within the ER gene appear rare, polymorphic variation in the ER gene may predispose to osteoporosis. This chapter therefore examines the association between ERα gene polymorphisms and bone-related traits. Because of the importance of oestrogen in the attainment of peak bone mass and the bone loss that occurs in the oestrogen-deficient state, the polymorphisms were examined against pre- and postmenopausal bone mass and early menopausal bone loss at both the lumbar spine and hip.

VI.2.2 Methods

Two groups of women were selected from the Chingford study population cohort. Group 1 consisted of postmenopausal women who were within 5 years of a natural menopause and currently not on HRT or other medication known to affect bone metabolism. Longitudinal BMD data were available on these women over 5 years. Group 2 consisted of premenopausal women who had regular menstruation, absence of menopausal symptoms and no current use of HRT.

ERα genotypes were determined as described in Chapter II using PCR and restriction enzyme digestion with *PvuII* and *XbaI*. *TaqI* VDR genotypes were also available on these subjects as previously detailed. Chi-squared analysis was performed to test for LD between the two ER polymorphisms. Haplotype analysis was subsequently performed, with direct comparisons between the variable means of the common haplotype combinations using two-sided Student *t*-test.

VI.2.3 Results

Full results were available on 202 women at the *PvuII* locus and 193 women at the *XbaI* locus. In total, 188 of the women had genotypic information at both loci. The genotype distribution at these two loci in these 188 women is shown in Table VI.1 below, and demonstrates strong LD between the loci ($\chi^2 = 59.4$, $P < 0.001$).

Table VI.1 **Distribution of subjects (%) at the *PvuII* and *XbaI* loci in 188 women**

	PvuII locus		
XbaI locus	PP	Pp	pp
XX	22 (11.7)	12 (6.4)	4 (2.1)
Xx	22 (11.7)	64 (34.0)	10 (5.3)
xx	5 (2.7)	21 (11.2)	28 (14.9)

Table VI.2 **Oestrogen receptor gene haplotypes in 188 women**

	PvuII genotype		
XbaI genotype	PP	Pp	pp
XX	2, 2	2, 3	3, 3
Xx	1, 2	-	3, 4
xx	1, 1	1,4	4, 4

The distribution of genotypes did not differ significantly between the groups of pre- and postmenopausal women.

With evidence of LD between the two loci, it was possible to assign 4 haplotypes: Px = 1, PX = 2, pX = 3, px = 4. In the absence of parental data and that the fact that each genotype was assigned independently, inference was made of the haplotypes for all subjects apart from the cases where subjects were heterozygous at both the *PvuII* and *XbaI* loci (Pp/Xx). Haplotypes 2 and 4 appeared the most common in this group of Caucasian women (Table VI.2).

Characteristics of the women according to their *PvuII* and *XbaI* genotypes are shown in Tables VI.3 and VI.4 respectively. There was no association between ER genotypes and bone mass at spine or hip, both in the total group and after stratification by menopausal status. In addition, there was also no association with rates of early postmenopausal bone loss at these sites. Non-significant findings were also observed with haplotypic analysis against these traits (Figures VI.1).

There was no evidence of interaction between the *TaqI* VDR gene polymorphism and each of the ER polymorphisms on BMD or rates of change in BMD (Tables VII.5 and VII.6), or when the analysis was repeated using constructed ER haplotypes.

Table VI.3 **Mean characteristics (SD) of 202 women according to *PvuII* genotype**

Characteristic	*PvuII* genotype		
	PP (n = 51)	Pp (n = 107)	pp (n = 44)
Age (yrs)	50.8 (5.0)	50.5 (4.1)	50.7 (4.5)
Height (cm)	162 (5)	162 (6)	161 (7)
Weight (kg)	65.9 (10.4)	68.6 (13.9)	67.9 (12.6)
BMI (kg/m^2)	25.0 (3.8)	26.0 (5.3)	26.2 (4.2)
No. subjects postmenopausal (%)	25	48	22
Menopause duration (yrs)	2.8 (2.1)	2.9 (1.6)	3.9 (3.2)
Age at menopause (yrs)	51.8 (3.2)	50.2 (3.1)	50.4 (3.1)
No. ever smoking (%)	26	59	22
Premenopausal lumbar spine BMD (g/cm^2)	1.05 (0.12)	1.07 (0.13)	1.05 (0.08)
Postmenopausal lumbar spine BMD (g/cm^2)	0.98 (0.17)	0.96 (0.14)	0.98 (0.12)
Premenopausal femoral neck BMD (g/cm^2)	0.80 (0.11)	0.82 (0.11)	0.82 (0.09)
Postmenopausal femoral neck BMD (g/cm^2)	0.76 (0.17)	0.77 (0.12)	0.80 (0.12)
Change in lumbar spine BMD (%/yr)	-1.1 (1.1)	-0.7 (1.6)	-1.0 (1.6)
Change in femoral neck BMD (%/yr)	-0.7 (2.0)	-0.6 (2.2)	-0.6 (2.3)

Table VI.4 **Mean characteristics (SD) of 193 women according to *XbaI* genotype**

	XbaI genotype		
Characteristic	XX (n = 38)	Xx (n = 100)	xx (n = 55)
Age (yrs)	50.3 (4.5)	50.5 (4.1)	50.7 (4.7)
Height (cm)	162 (5)	162 (6)	163 (7)
Weight (kg)	66.4 (12.4)	67.1 (13.2)	69.4 (12.3)
BMI (kg/m^2)	25.3 (5.2)	25.6 (4.9)	26.2 (4.2)
No. subjects postmenopausal (%)	14	52	32
Menopause duration (yrs)	3.7 (2.1)	3.1 (2.6)	2.9 (1.7)
Age at menopause (yrs)	51.7 (2.0)	50.7 (3.1)	51.3 (3.3)
No. ever smoking (%)	17	41	24
Premenopausal lumbar spine BMD (g/cm^2)	1.06 (0.12)	1.07 (0.13)	1.05 (0.09)
Postmenopausal lumbar spine BMD (g/cm^2)	0.98 (0.13)	0.96 (0.17)	0.98 (0.11)
Premenopausal femoral neck BMD (g/cm^2)	0.79 (0.12)	0.83 (0.11)	0.83 (0.10)
Postmenopausal femoral neck BMD (g/cm^2)	0.77 (0.13)	0.78 (0.15)	0.77 (0.12)
Change in lumbar spine BMD (%/yr)	-0.9 (0.9)	-0.9 (1.7)	-0.8 (1.5)
Change in femoral neck BMD (%/yr)	-0.5 (2.8)	-0.8 (2.0)	-0.5 (1.9)

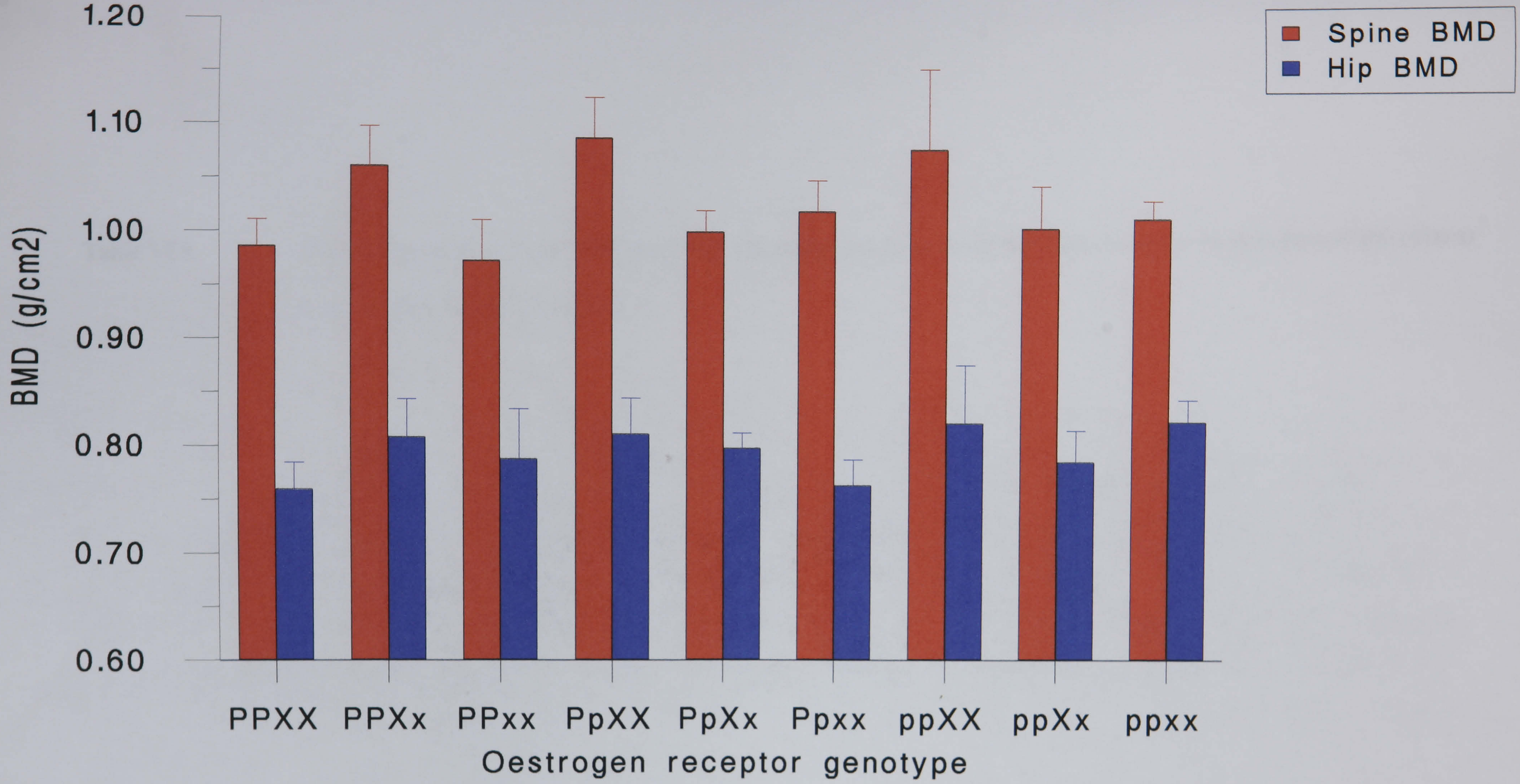


Figure VI.1 **Mean (± SE) BMD for ER genotypes at the lumbar spine and femoral neck**

Table VI.5 **Interaction between *TaqI* VDR and *PvuII* ER genotypes on mean BMD (SD) and mean % change in BMD (SD) at lumbar spine and femoral neck**

	Genotype								
	TTPP	TTPp	TTpp	TtPP	TtPp	Ttpp	ttPP	ttPp	ttpp
Lumbar spine BMD (g/cm2)	1.08 (0.14)	0.98 (0.16)	0.99 (0.13)	0.97 (0.11)	1.02 (0.11)	1.04 (0.08)	0.98 (0.17)	1.02 (0.16)	0.98 (0.11)
Femoral neck BMD (g/cm2)	0.82 (0.15)	0.77 (0.11)	0.80 (0.16)	0.74 (0.12)	0.80 (0.12)	0.89 (0.08)	0.82 (0.11)	0.79 (0.11)	0.81 (0.10)
Change in spine BMD (%/yr)	-1.19 (1.06)	-0.61 (1.74)	-1.70 (1.54)	-1.01 (1.27)	-0.73 (1.41)	-0.74 (1.51)	-1.35 (0.79)	-0.54 (1.64)	-1.06 (1.22)
Change in hip BMD (%/yr)	-0.42 (1.25)	-0.01 (1.48)	-1.26 (1.78)	-0.92 (2.23)	-1.27 (2.76)	-0.40 (2.23)	-0.91 (3.12)	-0.84 (2.13)	-1.00 (1.97

Table VI.6 **Interaction between *TaqI* VDR and *XbaI* ER genotypes on mean BMD (SD) and mean % change in BMD (SD) at lumbar spine and femoral neck**

	Genotype								
	TTXX	TTXx	TTxx	TtXX	TtXx	Ttxx	ttXX	ttXx	ttxx
Lumbar spine BMD (g/cm2)	1.07 (0.12)	1.00 (0.18)	0.99 (0.12)	1.03 (0.12)	0.99 (0.12)	1.03 (0.08)	0.94 (0.12)	1.04 (0.16)	1.03 (0.13)
Femoral neck BMD (g/cm2)	0.76 (0.12)	0.80 (0.13)	0.81 (0.14)	0.82 (0.13)	0.78 (0.12)	0.78 (0.09)	0.76 (0.09)	0.82 (0.11)	0.83 (0.09)
Change in spine BMD (%/yr)	-1.08 (1.07)	-0.78 (2.09)	-1.09 (1.28)	-0.80 (1.03)	-0.87 (1.49)	-0.83 (1.54)	-1.07 (0.47)	-1.02 (1.40)	-0.62 (1.60)
Change in hip BMD (%/yr)	+0.36 (1.16)	-0.78 (1.82)	-0.70 (1.84)	-1.50 (3.6)	-0.84 (2.24)	-0.85 (2.20)	+0.65 (2.27)	-1.11 (2.21)	+0.38 (1.29)

VI.2.4 Discussion

The data presented in this chapter failed to show an association between two biallelic polymorphisms of the oestrogen receptor gene and osteoporosis. Specifically, no relationship was seen between ERα genotype and premenopausal bone mass, and between ERα genotype and early postmenopausal bone loss. Two common haplotypes were identified, although no relationship was seen when these were also analysed against the osteoporosis traits.

These findings contrast with other studies that have demonstrated association between ERα gene polymorphisms and BMD in postmenopausal women (Sano et al, 1995; Kobayashi et al, 1996), premenopausal women (Mizunuma et al, 1997; Willing et al, 1998), and males (Ongphiphadhanakul et al, 1998). Negative studies have, however, also been reported (Mizunuma et al, 1997; Gennari et al, 1998; Han et al, 1999), and there appears to be no consistent trend in either the genotype/haplotype associated with lower BMD or with the skeletal site affected in the associations studies reported (Table VI.7). The majority of studies have been conducted in subjects of Oriental descent, and further studies are required in Caucasians and Blacks to determine whether the ERα gene contributes to the racial differences that are observed in BMD and osteoporotic fracture risk. In addition, no study has examined for a relationship between ERα genotype and osteoporotic fracture, with the majority of studies concentrating on women in the pre-, peri-, and early postmenopausal years.

Table VI.7 **Summary of studies examining the relationship between ER gene polymorphisms and BMD**

Author	No. subjects (F/ M)	Menopausal status	RFLPs	Association
Sano 1995	144 (F)	Post	$(TA)_n$	$(TA)_{12}$: ↓ Z-score BMD LS / WB
Kobayashi 1996	238 (F)	Post	PvuII, XbaI	Px haplotype: ↓ Z-score BMD LS/ WB
Mizunuma 1997	43 (F)	Pre	PvuII, XbaI	Xx vs xx: ↑ LS BMD
Mizunuma 1997	130 (F)	Peri/ Post	PvuII, XbaI	NS: LS BMD
Willing 1998	253 (F)	Pre/ Peri	PvuII, XbaI	PP genotype: ↑ LS/WB BMD XX genotype: ↑ LS BMD
Gennari 1998	426 (F)	Post	PvuII, XbaI	NS: LS/FN BMD
Ongphiphadhanakul 1998	81 (M)	-	PvuII	PP/Pp vs pp: ↑ LS BMD
Han 1999	598	Pre/post	PvuII, XbaI	NS: LS/FN BMD

LS: Lumbar spine FN: Femoral neck WB: Whole body NS: non significant

In two of the studies summarised in Table VI.7, genotype associations were also observed with markers of bone turnover (Sano et al, 1995; Mizunuma et al, 1997), although direct assessment of change in BMD over 3 years failed to demonstrate any significant relationship (Willing et al, 1998). In this latter study, subjects were pre- and peri-menopausal and it therefore remains to be confirmed whether the ER gene has any effect on differential rates of bone loss in the postmenopausal period.

In this chapter there was no evidence of interaction between the ERα gene polymorphisms and the *TaqI* VDR polymorphism on BMD or rates of change in BMD. Studies analysing the *BsmI* VDR polymorphism have, however, shown an association between this and the ER *PvuII* polymorphism on spine BMD (Gennari et al, 1998; Willing et al, 1998) and rates of growth in childhood (Suarez et al, 1998). In the study by Willing et al (1998) there was, however, no evidence of interaction between the two loci acting on rates of change in BMD. Although there is strong evidence of LD between the *BsmI* and *TaqI* VDR polymorphisms (Morrison et al, 1994), the interaction between the VDR locus and ER gene may be specific for the *BsmI* polymorphism.

Limitations

The study design had approximately 70% power to detect either a difference of 0.5 SD in BMD or a 1 % difference in rates of change in BMD between the homozygous genotypes. For the study to have had 90% power ($\alpha = 0.05$), the total sample size needed would have been more than 300 subjects. By restricting analysis to premenopausal and to postmenopausal women not using HRT, the study could not

address the issue as to whether environmental factors such as exogenous oestrogen in the form of HRT may have interacted with ER genotype (Deng et al, 1998; Willing et al. 1998). In addition, it would be interesting to have analysed the relationship between ER genotype and the ages at menarche and menopause, parity, and to have examined for a possible interaction with use of the oral contraceptive pill.

The study design was also limited by the fact that the *PvuII* and *XbaI* polymorphisms were assessed individually. In the absence of parental data this meant that haplotypes could only be inferred, with the genotype PpXx not being able to be included in analyses. A more detailed assessment of ER gene polymorphisms including the TA repeat in the promoter region may be required to accurately define the sequence diversity in the gene and to assess the degree of LD across the gene. This will be particularly important as the *PvuII* and *XbaI* polymorphisms are intronic and their functional significance is unclear. Data from other candidate genes for cardiovascular disease have already highlighted the sequence diversity in coding and non-coding regions (Rieder et al, 1999) and the potential for haplotype analysis (Keavney et al, 1998). The recent discovery of the ERβ gene (Enmark et al, 1997) and polymorphic association with BMD in Japanese women (Ogawa et al, 2000) is also of interest. These data suggest that the the ERβ gene should be further examined for its relationship with BMD and fracture risk in larger groups.

Because of the age of the subjects very few of them had experienced osteoporotic fracture thereby precluding formal analysis. In fact, only 8 subjects had sustained such fractures and these were evenly distributed across the ERα genotypes.

VI.2.5 Conclusions

In this chapter the ER gene has been evaluated as a candidate gene for the development of osteoporosis. Pre- and postmenopausal women (within 5 years of a natural menopause) were included in this analysis. Genotype information was available for the *PvuII* and *XbaI* ER gene polymorphisms and the *TaqI* VDR polymorphisms. There was no evidence of association between the ER RFLPs and BMD at spine or hip in both the pre- and postmenopausal women. In addition, there was no relationship between these RFLPs and longitudinal assessment of bone loss at both skeletal sites. The study identified two common ER haplotypes (PX and px), although there was no haplotypic association with BMD or bone loss. There was also no evidence of interaction between the VDR and ER loci. These data would therefore suggest that the ERα gene is not a major candidate gene for the development of osteoporosis, and the results would preclude a significant locus effect on BMD and early menopausal bone loss. Further studies in older subjects will be required to determine whether this locus has any effect on fracture risk.

VI.3 Type I Collagen α1

VI.3.1 Introduction

Type I collagen is the major protein constituent in bone and in a few individuals with severe osteoporosis, coding sequence defects similar to those observed in

osteogenesis imperfecta have been demonstrated (Spotila et al, 1991). These structural mutations are, however, rare and unlikely to account for more than a small minority of the clinical cases seen (Spotila et al, 1994).

During the course of this thesis, work from Grant et al (1996) demonstrated an association between a COLIA1 polymorphism and both low BMD and vertebral fracture risk in two UK patient groups. In this chapter, the relationship between this COLIA1 polymorphism and BMD, osteoporotic fracture risk and biochemical markers of type I collagen resorption has been examined in a cross-sectional analysis of women from a separate UK population cohort.

VI.3.2 Methods

The study design was a nested case-control study selected from the Chingford study population cohort. Cases were subjects with either (i) self-reported personal history of appendicular fracture in the 10-year period preceding the study's onset (1978-1988), or (ii) prevalent vertebral deformity consistent with fracture on lateral thoracolumbar spinal radiographs. Controls had no fracture history and no radiographic evidence of vertebral fracture.

BMD data were available at the lumbar spine and hip in all subjects. COLIA1 and *TaqI* VDR genotype results were available as previously detailed in Chapter II. As previous work had suggested a dominant pattern of risk associated with the rarer "s" allele

(Grant et al, 1996), statistical analysis was planned to combine the "Ss" and "ss" genotype groups.

In a subgroup of women from the total cohort population urinary collagen cross-links pyridinoline and deoxypyridinoline results were available for analysis against COLIA1 genotype. Dr. Ian James from St. Bartholomew's Hospital London had performed these assays on fasting early morning urine samples. The urinary collagen cross-links were assayed using ion-pair reversed-phase high-performance liquid chromatography (HPLC) in the presence of 1-octanesulphonic acid (James et al. 1990). Inter and intra assay variations were less than 10% for both pyridinoline and deoxypyridinoline. This assay had been validated against standard gradient systems. where correlations of 0.95 (pyridinoline) and 0.92 (deoxypyridinoline) were observed between the two techniques in 27 normal women. Preliminary analysis suggested that urinary collagen cross-link values were not normally distributed and for subsequent analysis natural logarithmic transformation was required to normalise the data.

VI.3.3 Results

Complete clinical and genotype data were available on 185, predominantly postmenopausal, women with a mean age (SD) of 54.3 (4.6) years. 55 women had evidence of validated prevalent fractures at either the lumbar spine or appendicular sites. Descriptive details of the fracture cases and controls are shown below in Table VI.8.

Table VI.8 Mean characteristics (SD) of 185 women according to prevalent fracture status

Variable	Fracture Cases (n=55)	Controls (n=130)
Age (yrs)	56.4 (5.2) *	53.4 (4.0)
BMI (kg/m^2)	26.7 (4.7)	25.6 (3.8)
Menopause duration (yrs) [medain, interquartile range]	8 (4, 12) *	4 (2,5)
No. subjects postmenopausal (%)	52 (95%)	122 (94%)
No. subjects with ever use of HRT (%)	15 (27%) *	6 (0.5%)
No. subjects ever smoking (%)	21 (38%)	59 (45%)
Lumbar spine BMD (g/cm^2)	0.88 (0.15) *	0.97 (0.15)
Femoral Neck BMD (g/cm^2)	0.70 (0.12) *	0.76 (0.13)

* $P < 0.0001$ (vs controls)

Table VI.9 Mean characteristics (SD) of 185 women according to COL1A1 genotype

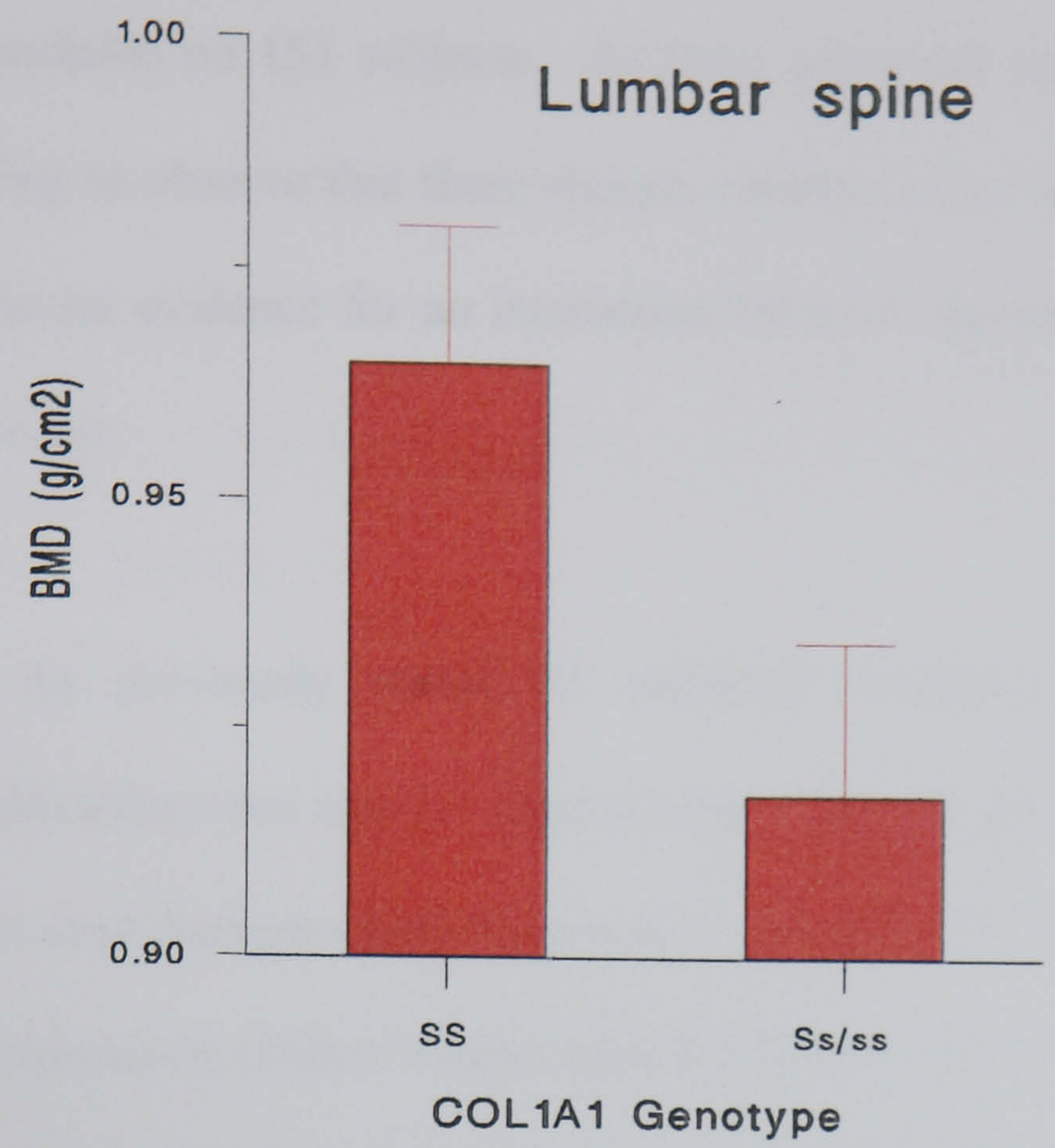
Variable	SS (n=113)	Ss (n=67)	ss (n=5)
Age (yrs)	54.0 (4.6)	54.8 (4.5)	55.0 (6.3)
Body Mass Index (kg/m^2)	26.0 (5.2)	25.8 (3.9)	26.4 (3.2)
Menopause duration (yrs) [median, interquartile range]	4 (2, 5)	4 (2, 8)	5 (4,5)
No. subjects postmenopausal (%)	106 (94 %)	63 (94 %)	5 (100%)
No. subjects with ever use of HRT (%)	11 (10 %)	9 (13 %)	1 (20%)
No. subjects ever smoking (%)	46 (41 %)	30 (45 %)	4 (80%)
No. subjects with fracture (%)	28 (25%)	27 * (40%)	0 (0%)
Lumbar spine BMD (g/cm^2)	0.965 (0.163)	0.912 (0.147)	0.924 (0.087)
Femoral neck BMD (g/cm^2)	0.754 (0.146)	0.726 (0.105)	0.757 (0.097)

* P = 0.03

In total, twenty-one women reported previous or current use of HRT, with a median (interquartile range) duration of therapy of 9 (3, 12) months. BMD was significantly reduced in the fracture cases compared to those without any history of prevalent fracture at both the spine and the hip.

The COLIA1 genotype frequencies observed in the total group were similar to those previously reported in white Caucasoids (Grant et al, 1996) and were in HWE: SS 61.1%, Ss 36.2%, ss 2.7%. No significant differences in demographic characteristics were observed between the women in the COLIA1 genotype groups (Table VI.9).

Analysis of the relationship between COLIA1 genotype and BMD showed that BMD was lower in heterozygous subjects ("Ss") at both the spine and the hip when compared to the homozygous genotypes "SS" and "ss" (Table VI.9). A pattern suggesting a dominant genetic effect associated with presence of the "s" allele was observed only at the spine. BMD was significantly reduced at the lumbar spine in subjects with the "s" allele ("Ss/ss" combined vs "SS"), mean difference (95% CI) 0.047 g/cm^2 (0.001, 0.093), P = 0.02 (Figure VI.2). A similar trend was also seen at the femoral neck, although this difference was non-significant, mean difference 0.026 g/cm^2 (-0.013, 0.065), P = 0.10.



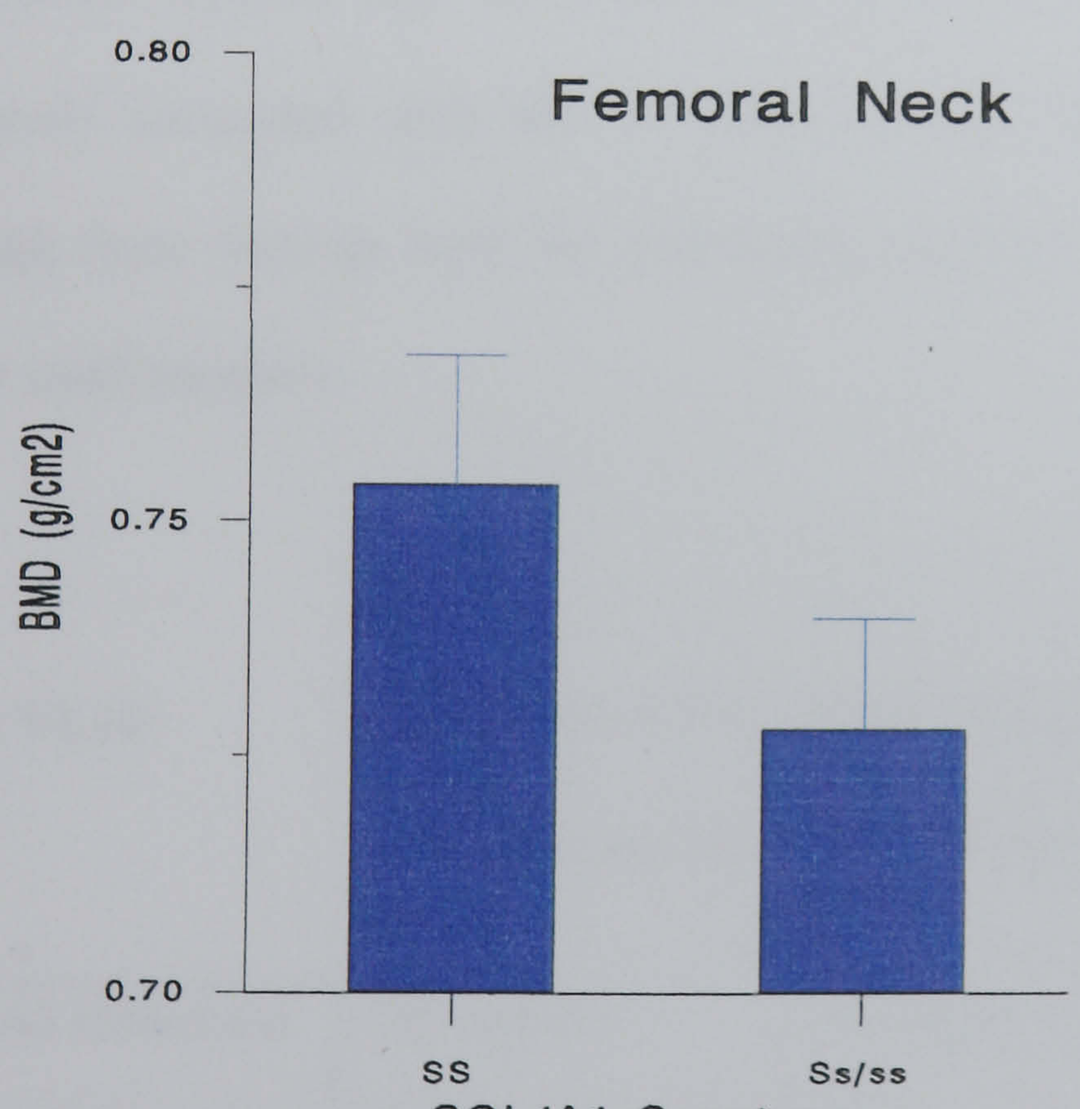


Figure VI.2 **Relationship between COLIA1 genotype and mean BMD (± SE) at the spine and femoral neck**

In total, full genotype results at both the COLIA1 and VDR loci and BMD data were available on 151 subjects. As these genes are on different chromosomes it was reassuring to observe that there was no evidence of co-segregation of genotypes. There was also no evidence for an interaction between the loci acting on BMD at either the spine or hip.

As previously stated 55 validated fractures were observed; 28 vertebral deformities/fractures and 27 appendicular. The risk genotypes ("Ss/ss") were higher in the prevalent fracture cases compared to non-fracture cases, with frequencies of 49% vs 33% respectively (Fisher's exact tests P = 0.04). The "Ss/ss" genotypes were associated with an increased risk of any fracture compared to the homozygous "SS" (Table VI.10). Site-specific analysis also demonstrated a consistent trend of fractures being more commonly associated with the "s" allele at both the spine and appendicular sites, although these findings were not statistically significant for the individual skeletal sites due to small numbers.

Table VI.10 **Odds ratio (95% CI) for risk of prevalent fracture vertebral and appendicular) associated with carriage of the "s" allele**

COLIA1 Genotype	OR (crude)	OR (adjusted *)
SS	1.00	1.00
Ss/ss	1.95 (1.01, 3.78)	1.82 (0.91. 3.61)

* adjusted for femoral neck BMD

Stratification of results by *TaqI* VDR genotype identified a subset of women with the heterozygous genotype "Tt" who were at significantly increased risk of fracture, odds ratio 5.59 (1.53, 20.36). No increase in fracture risk associated with the carriage of "s" allele was observed for women of the homozygous VDR genotypes "TT" or "tt" (Figure VI.6). Adjustment for confounders such as age, weight, BMD or calcium intake did not appreciably alter this risk.

Mean values for urinary collagen cross-links in the COL1A1 genotype groups are presented in Table VI.11. Pyridinoline concentrations were significantly elevated in subjects with the "Ss/ss" genotypes compared to the homozygous "SS". Levels of deoxypyridinoline did not, however, differ significantly between the genotype groups. Again, there was no evidence of any interaction between the COLIA1 and VDR genes acting on the urinary collagen cross-links.

Table VI.11 Relationship of COL1A1 genotype to mean (SD) urinary collagen cross-links

Variable	SS (n = 48)	Ss/ss (n = 34)
Pyridinoline/creatinine (nmol/mmol)	44.6 (24.5)	54.1 (24.5) *
Deoxypyridinoline/creatinine (nmol/mmol)	13.3 (7.0)	15.0 (7.2)

* $P < 0.05$ (vs SS)

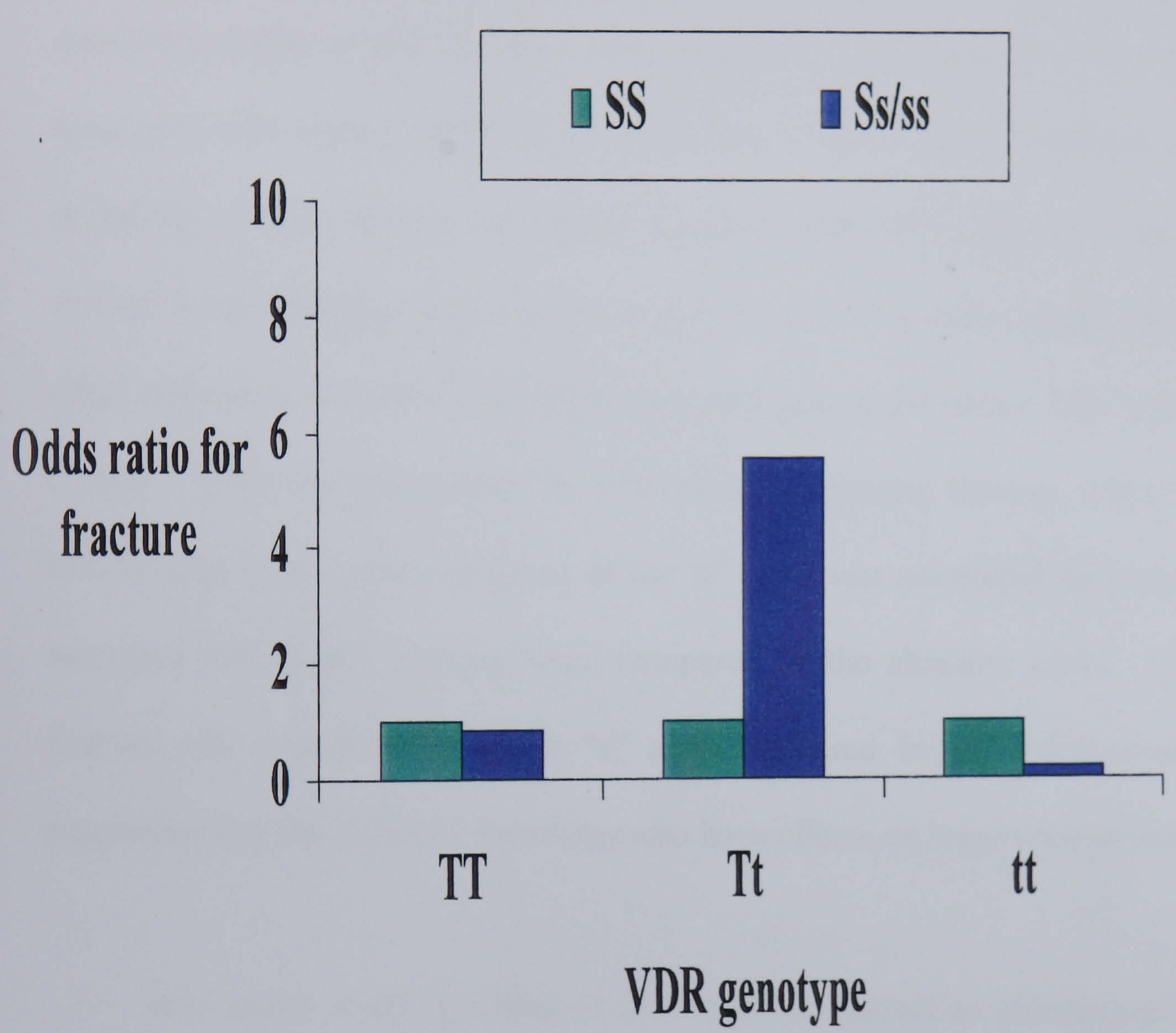


Figure VI.3 **Interaction between *TaqI* VDR and COLIA1 genotypes on total fracture risk**

VI.3.4 Discussion

Type I collagen is the major protein constituent of bone and is therefore a strong and plausible candidate gene for the regulation of BMD. The results presented in this chapter show an association between a single base pair polymorphism (G→T) within the regulatory region of the COLIA1 gene and risk of osteoporosis. The "s" allele was associated with reduced BMD at the spine with a more modest reduction also observed at the hip. These findings are similar to those observed in the two other populations studied from Aberdeen and London (Grant et al, 1996) and suggest that the genetic effect associated with the COLIA1 locus is strongest at the spine. The "risk" genotypes ("Ss/ss") were over-represented in subjects with prevalent fracture when compared to non-fracture cases, where presence of the "s" allele was associated with almost a 2-fold increased risk of any fracture when compared to the alternate allele. The increased fracture risk associated with the "s" allele appeared largely independent of BMD, suggesting that the COLIA1 locus may also have effects on bone structure or quality.

The earlier study by Grant et al (1994) had shown an association between the COLIA1 genotype and vertebral fracture (55 cases) with an almost 3-fold increased risk of fracture associated with the "Ss" and "ss" genotypes. The data from this chapter would indicate a consistent increased risk for any fracture although larger studies would be required for site specific analysis. During the course of this work other studies have also examined the relationship between the COLIA1 locus and osteoporosis. A study in 1,778 postmenopausal women (age range 55-80 years, mean age 66 years) from the Netherlands demonstrated a significant effect of the COLIA1 locus on BMD and fracture risk (Uitterlinden et al, 1998). In this study the COLIA1 locus explained only 0.3-0.4%

of the population variance in BMD with any effect only being apparent in subjects aged over 70 years. This may indicate some relationship between this polymorphism and age- or menopause related bone loss, and may also explain the preliminary observations from another cross-sectional study that the genetic effect on BMD associated with the COLIA1 locus was stronger in postmenopausal compared to premenopausal women (Garnero et al, 1998). Two smaller studies have, however, demonstrated an association between COLIA1 genotype and BMD in prepubertal girls (Sainz et al, 1999) and premenopausal women (Hampson et al, 1998). In the study by Uitterlinden et al (1998), carriage of the "s" allele was associated with an increased risk of peripheral fractures, although in contrast to the findings by Grant et al (1996) no increase in the risk of prevalent vertebral fractures was observed. This increase in fracture risk was not altered by adjustment for BMD, again perhaps suggesting some influence of this genotype on bone structure and quality. Data from other case-control studies was conflicting, with both positive (Langdahl et al, 1998; Roux et al, 1998) and negative (Liden et al, 1998; Hustmyer et al, 1999, Willing et al, 1997) associations being reported. It is interesting to observe that in the positive association studies there was no relationship between COLIA1 genotype and BMD, again suggesting a possible influence of this locus on bone quality or structure. To date, no studies have examined the relationship between the COLIA1 genotype and QUS parameters.

Case-control studies cannot confer a causal association between a genetic locus and a risk trait. The replication of the findings from Grant et al (1996) by work from this thesis and the other studies above (Uitterlinden et al, 1998; Roux et al, 1998; Garnero et al, 1998; Langdahl et al, 1998) provides stronger evidence and reduces the risk of type I

errors. Demonstration of a functional significance associated with the COLIA1 polymorphism which is located in a putative Sp1 binding site domain of the gene's promoter would strengthen the candidacy of this locus. The observation that pyridinoline levels were increased in the "at risk" genotype groups could suggest an association with collagen turnover. Deoxypyridinoline which is reported to be more specific for type I collagen resorbed from bone was not however significantly elevated in subjects with the "s" allele, suggesting that the COLIA1 locus rather than being bone specific, may have regulatory effects on total body turnover of type I collagen. This could indicate a possible relationship between this locus and type I collagen in skin, which would be of interest in view of the association observed in some studies between skin thickness and osteoporosis (Brincat et al, 1987; Pedersen et al, 1995). Langdahl et al (1998), however, found no relationship between urinary and serum markers of collagen turnover and COLIA1 genotype.

The data from the larger cross-sectional studies suggest a stronger effect of this locus on BMD with increasing age, and this could reflect an effect of the COLIA1 locus on differential rates of bone loss. This would be supported in part by the findings presented in this chapter demonstrating an association with collagen turnover. Longitudinal studies will be required to directly assess the relationship between the COLIA1 locus and menopausal bone loss.

The finding of a potential interaction between the COLIA1 and VDR locus is of interest. It appeared specific for the heterozygous genotype "Tt" rather than being associated with carriage of either the "T" or "t" alleles under different inheritance

patterns. The COLIA1 polymorphism is situated in a Sp1 binding site in the promoter region of the gene. The Sp1 transcription factor gene maps to the same chromosomal region as the VDR gene on chromosomal 12, and there is therefore a plausible biological hypothesis for an interaction. Because of issues relating to power and multiple comparisons these findings would need to be replicated in further studies to exclude the possibility of type 1 error. Environmental factors may also needed to be taken into account, although in this analysis results remained significant after inclusion of dietary calcium intake in the logistic regression model.

To date, only one study has presented genotype information for the two loci in the same population (Willing et al, 1997). In total, 66 women from the lower quartile of hip BMD measurements and 63 from the upper quartile were examined. There was no association between femoral neck BMD and either COLIA1 or VDR genotypes when analysed independently. No data was presented, however, for whether there was any interaction between the two loci.

VI.3.5 Conclusions

These data demonstrate that the COLIA1 gene polymorphism is associated with reduced spinal BMD and an increased risk of prevalent fracture. The increase in fracture risk was largely unaltered after adjustment for BMD, suggesting the locus may also have effects on bone structure or quality. Urinary pyridinoline, a marker of type 1 collagen resorption, was elevated in subjects with the risk genotypes "Ss" and "ss". There was evidence of interaction between the COLIA1 and VDR loci acting on fracture risk, although this was not seen for either BMD or markers of collagen turnover. The functional significance of the COLIA1 polymorphism remains to be determined. Longitudinal studies will be required to evaluate whether this gene could have a potential clinical role in the identification of a subset of women genetically predisposed to accelerated menopausal bone loss and/or increased fracture risk in later life.

VI.4 Interleukin 1 receptor antagonist

VI.4.1 Introduction

Oestrogen deficiency is associated with an increase in local production of various cytokines and growth factors within the bone marrow and bone cells, and these inflammatory factors appear to play an important role in the development of postmenopausal osteoporosis (Ralston, 1994). IL-1 is a powerful stimulant of bone resorption and is recognised as an inhibitor of bone formation (Dinarello, 1991). The observed postmenopausal increase in IL-1 activity results from an effect of oestrogen on the production of both IL-1 and the IL-1 inhibitor, IL-1ra. These and other data therefore raise the possibility that the interleukin 1 receptor antagonist gene is a potential candidate gene for the genetic regulation of early postmenopausal bone loss. In this chapter, therefore, the relationship between bone mass, early postmenopausal bone loss and an IL-1RN VNTR has been studied.

VI.4.2 Methods

From the Chingford study population cohort, 125 women were selected for the present study if they were within 5 years of a natural menopause and at their baseline examination were currently not on HRT or other medication known to affect bone metabolism. Longitudinal measurements of BMD over 5 years were available on subjects at spine and hip.

IL-1RN and *TaqI* VDR genotype results were available as previously detailed in Chapter II. For analysis, the IL-1RN alleles were collapsed into a biallelic system based on the number of repeats. The IL-1RN genotype A1 (4 repeats) was grouped with genotype A3 (5 repeats), and compared with genotypes A2 (2 repeats) and A4 (3 repeats). This reduces the problems associated with small numbers of subjects in several genotype groups, and has a plausible biological basis as the larger alleles will have probably arisen because of mutation from a common ancestral allele with fewer repeats. Where ANOVA values were significant ($p < 0.05$), the Bonferroni test for multiple comparisons was used to search for evidence of differences between individual genotype groups. Analysis for allele specific effects was performed using carriage of the collapsed allele classes under dominant and recessive risk models, with comparisons between the variable means using two-sided Student *t*-test.

VI.4.3 Results

Characteristics of the 125 women entered into the study are shown in Table VI.12. Full clinical and genotype results were available on 108 of these women. No significant differences were observed between subjects with genotype results and those where DNA was either unavailable or attempts at PCR unsuccessful.

Table VI.12 **Mean characteristics (SD) of study population and stratification by IL-1RN genotype**

	Total group (N=125)	A1A1 (n=45)	A1A2 (n=49)	A2A2 (n=7)	A1A3 (n=3)	A2A3 (n=4)
Age (yrs)	53.1 (3.5)	52.2 (3.4)	53.2 (4.0)	52.6 (2.8)	53.0 (4.0)	55.8 (1.5)
Age at menopause (yrs)	50.4 (3.1)	49.8 (3.1)	50.2 (3.9)	50.6 (3.4)	50.0 (4.0)	51.5 (1.0)
Menopause duration (yrs)	2.7 (1.7)	2.4 (1.8)	3.0 (1.6)	2.0 (1.8)	3.0 (0.0)	4.3 (0.5)
BMI (kg/m^2)	26.0 (4.8)	26.4 (4.7)	26.1 (5.3)	25.4 (3.9)	24.7 (3.1)	23.8 (2.1)
No. ever smoking (%)	54 (43)	20 (43)	23 (47)	0 (0)	1 (33)	3 (75)
Lumbar spine BMD (g/cm^2)	0.969 (0.144)	0.960 (0.145)	0.985 (0.139)	0.888 (0.118)	0.982 (0.082)	0.912 (0.123)
Femoral neck BMD (g/cm^2)	0.763 (0.130)	0.748 (0.129)	0.775 (0.131)	0.699 (0.100)	0.741 (0.170)	0.743 (0.110)
Change in lumbar spine BMD (%/yr)	-1.1 (1.5)	-1.4 (1.5)	-0.7 (1.5)	-1.6 (0.8)	-1.8 (0.5)	-0.9 (1.9)
Change in femoral neck BMD (%/yr)	-0.50 (2.2)	-0.9 (2.3)	0.0 (2.2)	-1.2 (0.9)	-0.4 (1.4)	-2.0[a] (-)

[a] only 1 subject in this group with annual rate of hip loss data

Analysis of the genotypes in the 108 women showed evidence of three alleles which corresponded both in size and in frequency to those previously identified (Table VI.13). From these three observed alleles, five out of the six possible genotypes were seen (Table VI.12). There was no significant difference between these 5 genotype groups in anthropomorphic or environmental variables known to influence bone mass.

Table VI.13 IL-1RN alleles identified through DNA amplification of 108 unrelated individuals (comparison with published data)

Allele	Size (bp)	Number of repeats (n)	Allele frequency (%) [a]	Allele frequency (%) [b]
A1	410	4	65.8	73.6
A2	240	2	31.0	21.4
A3	500	5	3.2	3.6
A4	325	3	-	0.7
A5	595	6	-	0.7

[a] Current study [b] Tarlow et al, 1993

Collapsing of the IL-1RN VNTR into a biallelic system gave rise to three genotype groups: A1A1 and A1A3 (n = 48), A1A2 and A2A3 (n = 53), and A2A2 (n = 7). No effect of IL-1RN genotype class was observed on baseline BMD at either the

lumbar spine or femoral neck. There was, however, a significant association between rates of change in BMD at the lumbar spine and the collapsed IL-1RN genotypes. This remained significant after adjustment for age, weight and baseline BMD. and also after adjustment for multiple testing using the Bonferroni test statistic. Subjects who were heterozygous for the A2 allele (genotypes A1A2 and A2A3) tended to have reduced rates of spinal bone loss when compared to subjects with the genotypes A1A1 and A1A3 (Bonferroni adjusted P value = 0.06). There were, however, too few subjects with the genotype A2A2 to observe a clear allele dose effect across the genotype groups.

Combining the A2 heterozyous and homozygous genotype groups, demonstrated that presence of at least one copy of the A2 allele was associated with reduced spinal bone loss (mean rate of change ± SD = -1.38 ± 1.48 %/yr) when compared to non-carriage of the A2 allele (mean rate of change = -0.81 ± 1.46 %/yr), $P < 0.05$ (Figure VI.4). Similar, but non-significant, trends were also seen at the femoral neck with reduced rates of bone loss associated with carriage of at least one copy of the A2 allele, (-0.85 ± 2.25 %/yr vs -0.23 ± 2.12 %/yr, P = 0.18). These findings were essentially unaltered after adjustment for the potential confounders as listed above.

VDR *TaqI* and IL-1RN genotype results were available on 99 subjects. There was no evidence of an interaction between the loci acting on either BMD or with rates of change in BMD.

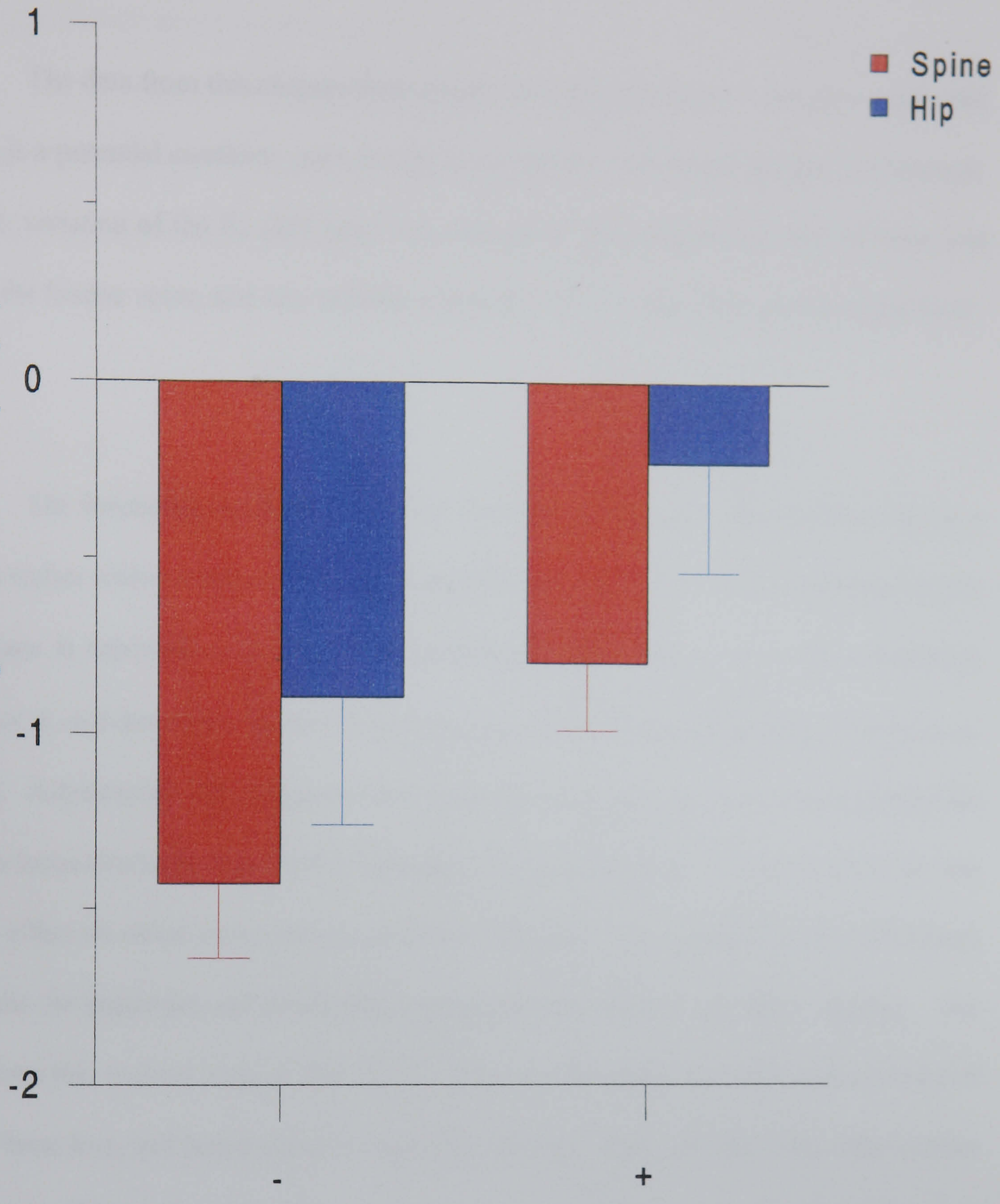


Figure VI.4 **Relationship between carriage of the IL-1RN A2 allele and mean bone loss (± SE) from the lumbar spine and femoral neck**

VI.4.4 Discussion

The data from this chapter demonstrate that the interleukin 1 receptor antagonist locus is a potential candidate gene for the development of postmenopausal osteoporosis. Allelic variation of the IL-1RN gene was associated with differential rates of bone loss from the lumbar spine, and may indicate a specific effect of this locus on trabecular bone.

The functional significance of these findings is not clear. The VNTR is due to a 86 bp repeat within intron 2 of the gene and this may be of functional significance as the sequence is reported to contain three potential protein binding sites; an α-interferon silencer A, a β-interferon silencer B and an acute phase response element (Tarlow et al, 1993). Polymorphic VNTR repeats have been shown to influence gene expression at the insulin locus (Vafiadis et al, 1997), although it is unknown if the IL-1RN VNTR has any direct effect on either osteoclast or osteoblast function. The physical number of repeats may also be important and could affect transcriptional activity and RNA stability. The data from this analysis suggest that the A2 allele may be associated with reduced rates of spinal bone loss, and hence reduced osteoclast activity. This contrasts with other studies that have shown the A2 allele to be associated with disease severity in several inflammatory diseases (Blakemore et al, 1994; Mansfield et al, 1994; Blakemore et al, 1995). One possible explanation could relate to the menopausal status of subjects as many of the disease associations have been documented in younger subjects. In the analysis presented in this chapter, the study was restricted to postmenopausal women and this may suggest that the A2 allele is specifically associated with reduced

inflammatory activity in the oestrogen deficient state. Further studies will need to examine whether there is allele specific expression of IL-1ra that is modified by hormonal status. In the absence of functional data on IL-1ra activity, however, an alternative hypothesis is that the IL-1RN VNTR is in LD with a disease causing gene nearby on chromosome 2, and that the degree of LD could have differed between the patient groups studied.

To date, fewer studies have examined cytokine polymorphisms for their relationship with osteoporosis and associated risk traits than has been seen with the VDR and COLIA1 loci. A CA microsatellite repeat polymorphism of the interleukin 6 gene has been associated with BMD in two separate studies (Murray et al, 1997; Tsukamoto K et al, 1999). Replication of study findings is important in genetic association studies to confirm original findings and exclude type I errors.

Limitations

The subjects represented in this analysis had a mean age of 53 years and would have already experienced some age related bone loss. The lack of an association between IL-1RN genotypes and baseline BMD suggests that the genetic effect of the locus on osteoporosis risk is not mediated through a major effect on peak BMD although testing specifically in premenopausal subjects would provide confirmation. Longitudinal epidemiological studies show continued bone loss in elderly subjects (Jones et al, 1994), and it remains to be determined if the demonstrated association between the IL-1RN

locus and spinal bone loss persists into later life. This could result in significant differences in BMD between the two common alleles in the elderly, translating into differential fracture rates. The design of the study to examine menopausal bone loss rather than fracture meant that a relationship with osteoporotic fracture could not be accurately assessed. The lack of an association at the hip may suggest separate genetic mechanisms at the different skeletal sites, although the greater variability in loss rates and the lower absolute values at this site could have precluded identification of any effect.

VI.4.5 Conclusions

In summary, these data demonstrate an association between a polymorphic VNTR in intron 2 of the IL1-RN gene and menopause related bone loss from the lumbar spine. A similar trend was observed at the femoral neck, although this failed to reach statistical significance. This suggests that this genetic locus may play a central role in the regulation of trabecular bone turnover in the oestrogen deficient state. There was no relationship between IL-1RN genotype and bone mass at either skeletal site, suggesting that this locus does not have a major influence on the attainment of peak BMD. There was also no evidence of interaction between IL-1RN and VDR *TaqI* genotypes acting on either BMD or rates of change in BMD at both spine and hip. Future studies should determine whether the relationship between IL-1RN genotype and rates of change in spine BMD extends beyond the first five years of the menopause and whether it translates into a permanent and significant difference in BMD in later life with increased fracture risk.

CHAPTER VII

VITAMIN D RECEPTOR GENOTYPE AND RISK OF OSTEOARTHRITIS

VII.1 Summary

Clinical observations suggest an inverse relationship between osteoporosis and osteoarthritis (OA), with OA cases having increased BMD and possibly a reduced fracture risk. As twin and family based studies have indicated a genetic predisposition to OA, this could support a hypothesis that osteoporosis and OA may share a common genetic aetiology. At the onset of this thesis, the VDR gene had been identified as a potential candidate gene for the determination of osteoporosis. This chapter has analysed whether the VDR *TaqI* polymorphism has any influence on the risk of OA at both the knee and spine in unrelated women. The hypothesis under test would predict that the allele associated with higher BMD would also be associated with an increased risk of OA.

Two nested case-control studies were performed in postmenopausal women. VDR genotype frequencies were found to differ significantly between knee OA cases and controls (Fisher's exact test $p = 0.03$), and between spinal OA cases and controls (Fisher's exact test $p = 0.02$). No relationship was observed, however, between VDR genotype and presence of Heberden's nodes on clinical examination. Carriage of the VDR "T" allele was associated with an almost 3-fold increased risk of OA at either knee or spine when compared to the "t" allele, with a dominant inheritance pattern of risk suggested. Findings were essentially unaltered after adjustment for confounders such as age, weight, and BMD, and after stratification for presence of OA at other sites.

These data suggest that VDR genotype is a marker for OA at both spine and knee, and this relationship is independent of any genotypic association with BMD. The data also highlight that other candidate genes may have influences on risks of osteoporosis and OA.

VII.2 Introduction

OA is a common age related and chronic skeletal condition associated with considerable morbidity and mortality, although at present the aetiology and pathogenesis of this condition remain largely unknown. OA of the knee and hip appears to be beneficial for bone mass, with OA cases having a 5-10% greater bone mass compared to controls (Dequeker, 1996). There is also evidence that OA is associated with underlying abnormalities in bone structure and mineralisation, findings that are independent of body weight and skeletal loading (Gevers et al, 1989). Family studies have suggested a strong genetic component to OA, with higher prevalence rates in first-degree relatives of index cases and familial aggregation (Kellgren et al, 1963; Felson et al, 1998; Hirsch et al, 1998). A recent population based UK twin study has also demonstrated a clear genetic effect for radiological OA at the knee and hand in women, with up to 65% of the variance being explained by genetic factors (Spector et al, 1996). Twin studies also support a significant genetic contribution to spinal degenerative change seen on magnetic resonance imaging (Battie et al, 1995; Sambrook et al, 1999).

As evidence exists for a strong genetic component to both OA and osteoporosis, this raises the possibility that common genetic factors may influence the development and/or protection against both conditions. To address this issue, this chapter examines whether allelic variation at the VDR locus may also be associated with an altered risk of OA in postmenopausal women and whether this risk is independent of BMD.

VII.3 Methods

VII.3.1 Subjects

The study design was to perform two nested case-control studies (knee OA and spinal OA) within postmenopausal women the Chingford Study population. BMD results and *TaqI* VDR genotype results were available as previously described in Chapter II.

VII.3.2 Knee and spinal radiographs

Antero-posterior weight bearing knee radiographs had been performed at baseline using standard procedures. Grading was performed blind by a single observer (Dr Debbie Hart) according to the methods of Kellgren & Lawrence where a scale of 0 to 4 is used (0 representing no disease and 4 representing severe disease) (Kellgren and Lawrence, 1963). Radiological definition of OA is the currently accepted standard for epidemiological studies of OA in populations (Spector et al, 1993). Knee OA was defined as present if a grade of 2 or more was given (definite osteophyte ± narrowing), and controls were classified as having no OA with a grade of ≤1. The intra-observer reproducibility for this technique was good with a kappa score of over 0.8. Presence of Heberden's nodes was determined by clinical hand examination (Egger et al, 1995).

Standard lateral radiographs of the thoracic (T4-12) and lumbar (L1-4) spine were also obtained at baseline using standard techniques and graded for presence of osteophytosis according to published criteria on a scale of 0 to 3: (0 = none, 1 = minimal, 2 = definite, 3 = severe). Spinal OA was defined as present in subjects with a grade ≥ 1 in at least one of the two levels scored (Orwoll et al, 1990).

VII.3.3 Statistical Analysis

Differences in demographic variables between OA cases and controls, and between VDR genotypes were initially compared using analysis of variance and chi-squared test. VDR genotype frequencies were compared between OA and control groups using Fishers exact test. Conditional logistic regression analysis was used to estimate the odds ratio and 95% test based confidence intervals for developing a radiological feature of OA for the individual VDR genotypes, with the homozygous VDR genotype "tt" set as baseline. Adjustment for other potential confounding variables was also performed using conditional logistic regression.

VII.4 Results

Relationship between *TaqI* VDR genotype and knee OA

In total, 501 women from the total cohort were postmenopausal and had graded knee radiographs. Full genotype results were available on 351 (70%) women: 82 subjects with knee OA and 269 with normal radiographs. The mean age ± SD for the total group of 351 women was 55.3 ± 5.0 years. No significant differences were observed between subjects with genotype results and those where DNA was unavailable.

The characteristics of the women according to the absence or presence of knee OA are shown in Table VII.1, and are similar to those of the whole cohort except for age, menopause status and duration. Within the 351 women with full results, there were significant differences between the cases and controls for potential confounders such as age, BMI, use of HRT, and hip BMD. Subdivision of women according to their

radiological grading for OA demonstrated that the majority of cases had minor disease: 63 (76.8 %) were grade 2, 15 (18.3 %) grade 3 and 4 (4.9 %) grade 4. For controls, 201 (74.7 %) were grade 0 and 68 (25.3 %) grade 1.

Overall, VDR genotype frequencies were in HWE and similar to those observed in other Caucasian groups and in the whole cohort. Table VII.1 shows that there were no significant differences in the baseline characteristics between the VDR genotypes within this total group or after stratification by Kellgren and Lawrence OA grade.

The frequencies of the *TaqI* genotypes were found to differ significantly between the OA cases and controls using Fisher's exact test (P = 0.03) with the "tt" genotype frequency being reduced with worsening OA status, although the number of subjects with severe OA (grade ≥ 3) was too small for formal analysis (Table VII.2).

The crude and adjusted odds ratios (95% CI) for knee OA in the "TT" and "Tt" VDR genotype group compared to the homozygous genotype "tt" are shown in Table VII.3.

Table VII.1 **Mean characteristics (SD) of women according to their knee OA status**

Variable	Controls	OA Cases					
	Total (n=269)	Grade 0 (n=201)	Grade 1 (n=68)	Total (n=82)	Grade 2 (n=63)	Grade 3 (n=15)	Grade 4 (n=4)
Age (yrs)	54.4 (4.8)	54.1 (4.8)	55.4 (4.6)	58.3* (4.7)	58.1 (4.6)	58.1 (5.2)	62.5 (1.9)
BMI (kg/m^2)	25.0 (3.6)	24.5 (3.5)	26.2 (3.8)	28.0* (4.0)	27.7 (3.7)	28.4 (4.5)	30.8 (4.9)
Age at menopause (yrs)	47.8 (5.0)	47.9 (4.9)	47.1 (5.3)	47.7 (4.7)	47.7 (4.8)	48.1 (4.8)	46.5 (4.1)
No. ever use of HRT (%)	78 (29%)	57 (28%)	21 (31%)	15 † (18%)	10 (16%)	5 (33%)	0 (0%)
No. ever smokers (%)	112 (42%)	83 (41%)	29 (43%)	37 (45%)	29 (46%)	6 (40%)	2 (50%)
BMD Femoral neck (g/cm^2)	0.73 (0.12)	0.73 (0.11)	0.75 (0.12)	0.77 ‡ (0.13)	0.77 (0.14)	0.78 (0.10)	0.72 (0.08)

* $P < 0.001$ (cases vs controls)

† $P = 0.05$ (cases vs controls)

‡ $P = 0.008$ (cases vs controls)

Table VII.2 **VDR genotype distribution (%) according to Kellgren and Lawrence OA Grade**

Genotype	Grade 0 (n = 201)	Grade 1 (n = 68)	Grade2 (n = 63)	Grade 3 (n = 15)	Grade 4 (n = 4)
TT	74 (36.8 %)	24 (35.3 %)	19 (30.2 %)	10 (66.7 %)	0 (0 %)
Tt	87 (43.3 %)	36 (52.9 %)	38 (60.3 %)	4 (26.7 %)	4 (100 %)
tt	40 (19.9 %)	8 (11.8 %)	6 (9.5 %)	1 (6.6 %)	0 (0 %)

Table VII.3 **Effect of VDR Genotype on Risk of Knee OA - Odds Ratio (95% CI)**

Genotype	Frequency in OA cases (%)	Frequency in controls (%)	OR (Crude)	OR (Adjusted *)
TT	29 (35.4 %)	98 (36.4 %)	2.42 (0.94, 6.21)	2.82 (0.98, 8.10)
Tt	46 (56.1 %)	123 (45.7 %)	3.15 (1.26, 7.83)	2.98 (1.09, 8.12)
tt	7 (8.5 %)	48 (17.9 %)	1.0	1.0

* Adjusted for age, BMI, femoral neck BMD, use of HRT

As risk of knee OA appeared to be associated with presence of the "T" allele with a dominant inheritance pattern, analysis was subsequently performed on the genotypes "TT" and "Tt" combined against the "tt" homozygous group set at baseline. Crude analysis showed an odds ratio of 2.82 (1.16-6.85, $p = 0.02$) for risk of knee OA in association with the "T" allele. This increase in risk remained after adjustment as previously detailed with an odds ratio of 2.60 (1.01-6.71, $p < 0.05$).

No significant relationship was observed between VDR genotype and nodal arthritis, as determined by the presence of Heberden's nodes. In addition there was no difference in genotype frequencies in the subset of knee OA cases who also had clinical nodes present compared to knee OA alone.

Relationship between *TaqI* VDR genotype and spinal OA

In total, 757 women from the total cohort were postmenopausal and had graded spinal radiographs. Full genotype results were available on 495 (65%) women: 104 subjects with spinal OA and 391 with normal radiographs. Site specific analysis showed that 63 subjects had evidence of OA changes in the thoracic spine only, 25 had changes only at the lumbar spine, with 16 subjects having changes at both levels. The characteristics of the women according to the absence or presence of spine OA are shown in Table VII.4, and are similar to those of the whole cohort except for age, menopause status and duration. No significant differences were observed between subjects with genotype results and those where DNA was unavailable.

Table VII.4 **Mean characteristics (SD) of women according to their spinal OA status**

	Controls (n = 391)	Spinal OA Cases (n = 104)
Age (yrs)	55.6 (5.2)	57.5 (5.1) *
BMI (kg/m^2)	25.1 (3.5)	27.3 (13.5) *
Menopause duration (yrs)	8.0 (6.0)	9.4 (5.9)
No. ever use of HRT (%)	103 (26%)	22 (21%)
No.ever smokers (%)	175 (45%)	48 (46%)
BMD Lumbar spine (g/cm^2)	0.93 (0.15)	0.98 (0.17) ‡
BMD Femoral neck (g/cm^2)	0.73 (0.12)	0.75 (0.12)

* $P < 0.001$ (cases vs controls)

‡ $P = 0.003$ (cases vs controls)

Again, overall the VDR genotype frequencies in this subgroup of 495 women were in HWE. There were no significant differences in the baseline characteristics as measured in Table VII.4 between the VDR genotypes within this total group or after stratification by presence of spinal OA.

The frequencies of the *TaqI* genotypes were found to differ significantly between the OA cases and controls using Fisher's exact test (P = 0.01). The crude and adjusted odds ratios (95% CI) for spinal OA in the "TT" and "Tt" VDR genotype groups compared to the homozygous genotype "tt" are shown in Table VII.5.

Table VII.5 Effect of VDR Genotype on Risk of Spinal OA - Odds Ratio (95% CI)

Genotype	Frequency in OA cases (%)	Frequency in controls (%)	OR (Crude)	OR (Adjusted *)
TT	37 (36 %)	139 (36 %)	2.62 (1.11, 6.19)	2.93 (1.16, 7.39)
Tt	60 (58 %)	183 (47 %)	3.23 (1.41, 7.41)	3.36 (1.34. 8.39)
tt	7 (6 %)	69 (17 %)	1.0	1.0

* Adjusted for age, weight, femoral neck BMD

Risk of spinal OA again appeared to be associated with presence of the "T" allele with a dominant inheritance pattern. Analysis was therefore subsequently performed on the genotypes "TT" and "Tt" combined against the "tt" homozygous group set at baseline. Crude analysis showed an odds ratio of 2.97 (1.32-6.67) for risk of spinal OA in association with the "T" allele. This increase in risk was marginally increased after adjustment as previously detailed with an odds ratio of 3.19 (1.32-7.72). This analysis was also performed after exclusion of subjects with prevalent knee OA. The risk of spinal OA associated with the "T" allele remained increased, with a crude odds ratio of 2.45 (1.01, 5.89). These findings were also essentially unaltered after adjustment for age, weight and BMD.

VII.5 Discussion

These results demonstrate associations between a *TaqI* polymorphism of the VDR locus and early knee OA and spinal OA in women from the general population. The "T" allele, which has previously been associated with high bone mass in some twin and population studies, is associated with a nearly 3-fold increased risk for development of OA at the two skeletal sites when compared to the alternate allele. The pattern of risk was observed to be co-dominant, with the homozygous genotype "TT" and the heterozygous genotype "Tt" both having an equivalent increased risk of OA. This relationship cannot be explained on the basis of age, as no significant deviation from the expected genotype frequencies was observed in the total group after stratification by age. This relationship was also independent of other factors such as weight and BMD that have been shown to influence OA risk and were found to differ between the cases and controls.

The finding that there was no relationship between the VDR locus and nodal arthritis also suggests that differing genetic mechanisms may underlie the development of Heberden's nodes and associated generalised OA. There may also be some evidence for site specificity, as the findings at the spine appeared independent of the observed association at the knee.

It has been proposed that the increase in bone density observed in OA indicates that the latter disease might initially be a subchondral bone disorder rather than a defect in cartilage, with an increase in subchondral stiffness (Radin et al. 1970). This would make the subchondral bone less deformable to impact loading and would result in more force being transmitted to overlying tissue, thereby predisposing to articular cartilage loss. The finding that there was no association between VDR genotype and BMD in this subset of women, and that the observed increase in OA risk associated with VDR genotype was independent of BMD suggests, however, that the molecular mechanisms are likely to be more complex than a simple direct action on bone strength with consequent cartilage damage.

Serum levels and dietary intake of vitamin D have been shown to correlate with progression of hip and knee OA, with this relationship being independent of any effect of vitamin D on BMD (McAlindon et al, 1996; Lane et al, 1999). Vitamin D has been shown to stimulate synthesis of proteoglycan by mature articular cartilage in vitro (Corvol et al, 1981), and this suggests that vitamin D, through its receptor, may directly affect articular cartilage metabolism. Vitamin D also has effects on the immune system and altered function of the VDR may result in immuno-modulation

(Hewison, 1992). This may be relevant as low levels of inflammation have been associated with knee osteoarthritis (Sipe, 1995; Spector et al, 1997). Unfortunately, serum measurements of vitamin D metabolites were not available on these subjects and it was therefore not possible to test this hypothesis that VDR genotype may cause alterations in vitamin D homeostasis that could result in an OA phenotype. Analysis was also not performed to test for an association between VDR genotype and serum markers of inflammatory activity such as C-reactive protein levels in these subjects.

The current study cannot exclude the possibility that the *TaqI* polymorphism may be in LD with a nearby novel susceptibility locus on chromosome 12 that has effects on both bone and cartilage metabolism. Potential candidate genes for the development of OA that map to this chromosomal region include the COL2A1 IGF-1 loci that have both been implicated in OA and osteoporosis (Dequeker et al, 1993; Bleasel et al, 1995). A genome-wide linkage scan for OA in 481 families with a least one affected sibling pair (disease status defined as prevalent joint replacement for OA) has recently failed to identify linkage with chromosome 12 (Chapman et al, 1999). A further study in 27 sibships with distal interphalangeal OA gave initial linkage to a marker mapping to 12q24, although further typing of microsatellite markers in the region failed to replicate these findings (Leppavuori et al, 1999). Linkage studies may, however, have limited power to detect loci having modest effects on disease risk (Risch and Merikangas, 1996) and association studies could offer a more powerful approach.

The COL2A1 gene maps <920 kb from the VDR locus, and mutations of this locus have been demonstrated in familial forms of OA and chondrodysplasias (Pedeutor et al, 1994). These mutations are, however, rare and unlikely to account for the majority of clinical cases observed in the general population. Several small association and affected sib pair (AS) linkage studies have failed to identify any association between the COL2A1 locus and OA in the general population (Hull et al, 1989; Vikkula et al, 1993; Priestly et al, 1991; Loughlin et al, 1994; Aerssens et al, 1998). A recent population-based study in 786 subjects aged 55 to 65 years has also identified an association between allelic variation at the IGF-1 locus and radiographic OA at several sites including knee, hip, hand, and spine (Meulenbelt et al, 1998).

During the course of this work, several studies have also been published that have examined the relationship between VDR genotype and OA. Uitterlinden et al (1997) examined 846 subjects (405 males, 441 females) aged above 55 years. Their results showed that carriage of the haplotype "baT" was associated with a 2-fold increased risk of OA, and is consistent with this Chapter's results. Earlier work from this group had. however, suggested that the "baT" haplotype was actually associated with reduced bone mass in this population so the pathophysiological mechanisms are uncertain (Uitterlinden et al, 1996). In the study reported by Aerssens et al (1998), no relationship was observed between *BsmI* genotype and hip OA. In this study disease status was defined by need for hip replacement and therefore represents a more severe, and probably more symptomatic, end of the disease spectrum. The negative findings at the hip may reflect site-specificity in the VDR-OA association, or that the gene influences susceptibility rather than severity of disease.

Two studies have specifically examined the relationship between VDR genotype and spinal OA. Jones et al (1998) studied spinal degenerative disease assessed on plain radiographs (L1-4) in 110 men and 172 women aged over 60 years (average age 69 years). Compared to the homozygous genotype "tt", subjects with the genotype "TT" had a 55% reduction in risk of disc space narrowing and a 59% reduction in the severity of osteophytosis. No data were available for OA at peripheral sites. Videman et al (1998) studied 85 pairs of MZ male twins (i.e. 170 subjects) aged 35 to 69 years with magnetic resonance imaging (MRI) of the thoraco-lumbar spine. Association analysis was performed using data for all subjects, taking account of the fact that MZ twins were being studied through the use of the generalised estimating equation. *TaqI* and *FokI* genotypes were both significantly associated with MRI measures of spinal degenerative change, with the "t" and "f" alleles associated with increased disease.

The concept that genes regulating BMD may also have an association with risk of OA is also supported by several studies. Polymorphisms of the oestrogen receptor gene have been associated with both osteoporosis (Sano et al, 1995; Kobayashi et al. 1996) and generalised OA (Ushiyama et al, 1998). In the ASP linkage study, an OA susceptibility locus was detected mapping to a region on chromosome 11q (Chapman et al, 1999). This region has also previously demonstrated significant linkage with a locus implicated in the regulation of bone mass from studies examining rare familial bone diseases (Gong et al, 1996; Johnson et al, 1997) and normal variation of BMD within sibships (Koller et al, 1998). The recent hand OA linkage study also provided evidence for linkage with chromosome 2q, a region harbouring the IL-1 gene family cluster

(Leppavuori et al, 1999). This suggests that the IL-1RN locus may also be a candidate for OA and warrants further study. Work is ongoing from these latter linkage studies to refine the linkage region and to then attempt positional cloning of the novel disease susceptibility loci.

Limitations

The study's results are limited to early knee and spinal OA in women as the number of subjects with severe OA was too small. At the knee, the majority of cases had osteophytosis with very few having significant loss of joint space. Additional, larger studies would be required to confirm if the observed relationship between VDR genotype and knee OA was dependent on differing genetic influences on either osteophyte development or cartilage loss. Similarly at the spine, the majority of cases had early disease as assessed by spinal radiographs. Improved phenotypic resolution would have been achieved with spinal MRI in this early disease state, although this was not available on subjects. Numbers were also too small for analysis to be performed on thoracic and lumbar spines separately.

The cross-sectional nature of the study design meant that prevalent OA was assessed and it was not possible to address issues on disease incidence or progression. Longitudinal studies would again be needed to determine whether this locus has any influence on the progression of joint damage at this site.

The finding of haplotypic association with knee OA (Uitterlinden et al, 1997), and association between the *FokI* alleles and spinal MRI characteristics (Videman et al. 1998) highlights the need for more extensive genotyping of the VDR gene. Haplotype analysis of a qualitative trait such as OA would be feasible, but large numbers of subjects would be required for adequate statistical power.

The data in this chapter demonstrated an association between *TaqI* VDR genotype and both spine and knee OA, although no association was observed between presence of Heberden's nodes. Although correlations exist between presence of OA at different sites, it is not clear if this equates to the same disease process. It is unclear from this analysis whether the observed associations at the spine and knee reflect a common genetic influence on the same pathological process or independent effects at the two sites. Statistical adjustment or stratification of analyses may support site-specificity, although multivariate modelling of data in a large number of MZ and DZ twins would provide a more powerful tool to address this issue (Martin et al, 1997).

VII.6 Conclusions

The data presented in this chapter have shown a relationship between early knee and spinal OA and a VDR *TaqI* polymorphism in unrelated women from the general population. The risk of OA was increased nearly 3-fold in those with the VDR genotypes "TT" and "Tt" when compared to those with the "tt" genotype. These findings suggest a dominant inheritance pattern of risk associated with carriage of the "T" allele. The risk of OA associated with VDR genotype was statistically independent of BMD, suggesting that the pathophysiological mechanisms underlying these results are likely to be more complex than a simple direct action on bone strength and consequent cartilage damage.

Findings in other populations have confirmed associations between the VDR gene and both knee and spinal OA, and further research should now be aimed at characterising the molecular basis to this relationship between the VDR protein and cartilage/bone metabolism. The data also suggest that candidate genes associated with osteoporosis may also have an important association with the development and risk of OA.

CHAPTER VIII

CONCLUSIONS AND FUTURE RESEARCH

VIII.1 Summary

The data presented in this thesis have confirmed that genetic factors appear to play an important role in determining both bone strength and fracture risk in women from the UK. The heritability estimates from the classical twin analysis suggest that up to 98 % of the population variance in total body BMD was accounted for by genetic factors. At specific skeletal sites the proportion of variance attributable to genetic factors ranged from 60 to 85 %, being highest in sites composed predominantly of trabecular bone. The population-based analysis used family history of fracture as a surrogate of genetic risk, and demonstrated a familial component to BMD and individual fracture risk. The data also suggested site-specificity in fracture risk, particularly at the wrist. In total, four candidate genes were analysed for association with BMD and related osteoporotic traits. Overall, the results suggested that the effect of individual genes on disease risk was modest, with potential and complex interactions between other candidate genes and environmental factors.

Extending the findings from this thesis would enable a more detailed insight into the genetic aetiology and architecture of osteoporosis. Modelling and path-based analysis of the twin data would allow a breakdown of the genetic components acting on BMD, examining for common or site-specific influences. In addition it would also enable the interaction between BMD and risk of OA to be further explored. For these types of analyses a larger number of twin subjects would be needed to have adequate power, and this forms the basis of ongoing research at St. Thomas' Hospital. Collection of further twins would also allow the heritability for fracture risk at individual sites to be estimated.

Assessment of subjects from the Chingford Study continues, with data collection up to 8 to 10 years. It should now be possible to determine the number of incident fractures that have occurred over this time period, and due to the age of the cohort it is anticipated that a large number of these will be of the distal forearm. It should then be possible to examine the relationship between family history of fracture and risk of incident fracture, thereby reducing the potential bias with assessment of prevalent fracture. Collection of additional longitudinal BMD measurements would also allow a more accurate estimation in rates of change in BMD, reducing the effects of the precision of the DXA machines. Candidate gene association analyses could be repeated against these extended and recalculated rates of change, although results may be statistically rather than clinically significant.

To explore further the relationship between OA and osteoporosis, additional genotyping of the Chingford cohort for the ER, IL-1RN and COLIA1 gene polymorphisms would be required. These genotypes were initially obtained on a subset of women from the total cohort, although the findings with the VDR gene suggest further analysis should be performed. With the availability of longitudinal OA data, it should also be possible to examine for genetic associations with either disease incidence or progression although power may be limited due to small numbers. With more comprehensive genotype information, the study could assess more accurately for gene-gene interactions. It may also be possible to examine for gene associations with response to therapy, particularly HRT.

Future work should also be directed towards examining other candidate genes in this cohort, thereby allowing further assessment of epistatic interaction between loci. During the course of this work a number of genes in addition to the four that have been described have been analysed for polymorphic association with BMD and fracture risk. These include transforming growth factor β1 (Langdahl et al, 1997), calcitonin receptor (Taboulet et al, 1998), apolipoprotein E (Shiraki et al, 1997), osteocalcin (Dohi et al, 1998), and parathyroid hormone (Hosoi et al, 1999). Many of these studies are small and have been conducted predominantly in subjects of Oriental descent. Confirmation of these findings in other ethnic and racial groups will be required in further studies.

At present choice of candidate genes has been determined by a prior knowledge of bone pathophysiology. The number of potential candidates will increase as scientific knowledge increases about the control and regulation of osteoclasts and osteoblasts, and their interaction with the bone matrix. Studies in knockout mice have identified bone phenotypes, suggesting important roles of these genes in bone metabolism and development (Iotsova et al, 1997; Kuro-o et al, 1997; Mizuno et al, 1998; Xu et al, 1998). Given the homology between the murine and human genomes, research is centred on examining whether the equivalent genes have similar effects in man. Identification of the gene defects responsible for rare familial bone diseases has also revealed additional candidates such as core-binding factor A1 (Mundlos et al, 1997) and cathepsin K (Gelb et al, 1996; Johnson et al, 1996). Recent quantitative trait linkage analysis studies in mice (Klein et al, 1998; Higuchi et al, 1999) and humans (Devoto M et al, 1998; Niu T et al, 1999) have also identified chromosomal regions that may harbour novel susceptibility loci for low BMD and osteoporosis. Interestingly, some of these

linkage regions in the human studies are consistent both between study populations and with homologous regions identified in the mouse studies. Further work will be required to isolate the susceptibility genes in these regions, although this will be expedited by progress in the Human Genome Project.

Once further candidate genes have been identified, it will be possible to screen for mutations and polymorphisms to assess the degree of genetic diversity. This has been undertaken for at least 175 candidate genes for cardiovascular disease (Cargill et al, 1999; Halushka et al, 1999), with future predictions that over 1,000 genes will have soon been analysed. It may then be possible to undertake large association studies with common allelic variants of multiple candidate genes (Kruglyak 1999). The challenge will be in interpreting the significance of results derived from this type of analysis. In addition, development of analytical methods will be required to explore the potential interactions between genes and environmental factors. Large collections of well-characterised population groups will also be necessary.

With an improved knowledge about the genetic determination of osteoporosis, what are the potential benefits to patients? Gene targets and molecular mechanisms may ultimately lead to novel drug discovery programmes, although this process may take some time to yield successful results. Knowledge that family history of fracture is an important clinical risk factor is important, and is recognised in guidelines for disease management and assessment with DXA. Accurate genotype information on several candidate genes may also aid identification of subjects at increased fracture risk, although the sensitivity and specificity for such assessments will need to be determined prior to

wide-spread clinical use. Gene polymorphisms that predict response to current therapies (i.e. vitamin D, HRT, bisphosphonates) may also allow the targeting of treatment to those most likely to respond and benefit. Gene therapy is also often highlighted as a potential goal for this type of research, although at present this remains some distance in the future.

In conclusion, this thesis has demonstrated a significant genetic contribution to osteoporosis and fracture risk in postmenopausal women. Future work may identify additional susceptibility genes and examine their influence on disease risk, thereby providing an improved understanding of the molecular mechanisms underlying the development of osteoporosis. This will hopefully ultimately lead to improved preventive, diagnostic and treatment options for subjects with osteoporosis.

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APPENDIX A

TWIN STUDY QUESTIONNAIRE

Draft

Twin Research Unit

GENERAL QUESTIONNAIRE - PART I

2.0 PRODUCTION STH

Study Type: T | Centre Code: STH | Study No: ____ - ____ | Date (Day/Month/Year): __ / __ / 19__

Interviewer Code: ___ | Visit Month: __ | Date Of Birth (Day/Month/Year): __ / __ / ____

A. About You

Surname

First Name(s)

Sex ○ Male ○ Female

Handedness ○ Right ○ Left ○ Ambidextrous

Ethnic Origin (Select one)

○ Caucasian ○ Asian ○ Oriental ○ Black ○ Other

Town of Birth

Country of Birth

---

hrs min

How long since you last ate or drank anything other than water? __ . __

GTT Time 0 - Time finished glucose drink (to be drunk within 5 minutes) __ . __

120 Time (minutes) ___

FSHA and Oestradiol ○ No ○ Yes

Testosterone ○ No ○ Yes

Draft

Study Type: T | Centre Code: STH | Study No: _____ - _____ | Visit Month: _____

B. You and Your Twin

Give Your Birth Weight in kg [ ] . [ ] [ ] [ ]

Were you born first or second?

- ○ First
- ○ Second
- ○ Third
- ○ Caesarian
- ○ Don't Know

Sister

- ○ Older than twins
- ○ Younger than twins

Was your birth weight heavier or lighter than your twin?

- ○ Heavier
- ○ Lighter
- ○ Same
- ○ Don't know

What type of delivery?

- ○ Normal
- ○ Caesarean
- ○ Premature (over 4 weeks)
- ○ Don't Know

One or two placentas?

- ○ One
- ○ Two
- ○ Three
- ○ Anything else or unknown or sister
- ○ N/A

C. Zygosity Score (score for each question given in parentheses)

In childhood, were you and your twin described as

- ○ As alike as peas in a pod (1)
- ○ Of ordinary likeness (2)
- ○ Don't Know (3)

At school, did people have trouble telling you apart?

- ○ Yes (1)
- ○ No (2)
- ○ Don't Know (3)

Who could tell you apart at school age?

- ○ Parents (1)
- ○ Brothers/Sisters (2)
- ○ Close school friends (3)
- ○ Strangers (4)
- ○ Don't Know (5)

Total [ ]

Zygosity

- ○ If total < 5 Monozygous
- ○ If total = 8 Dizygous
- ○ Unkown = 6, 7 and > 8

Did you share the same room as children?

- ○ Yes
- ○ No
- ○ Half of the time as child
- ○ Reared apart since birth

If yes, until what age did you share the same home? [ ] years

D. The Examination

Present height [ ] cms

Lifetime maximum height [ ] cms

Weight [ ] kg

The weight you were when aged 20 [ ] . [ ] kg

Lowest weight aged 20 or over [ ] . [ ] kg

Maximum weight you have been not including pregnancy [ ] . [ ] kg

Length of leg when measured on power rig [ ] . [ ] cms

Score on power rig (best of 3) [ ] . [ ]

Draft

T S T H

Initial systolic blood presure

Initial diastolic blood pressure

Repeat systolic blood presure

Repeat diastolic blood presure

Grip strength

best of 3 recordings on red score range

Dolorimeter score of the fore head

Body measurements

Onbreast cms

Underbreast cms

Waist cms

Hip cms

Thigh Measure. Measure the distance from where the leg meets the body down to the top of the knee cap and then half this distance and measure around the leg at this half way distance

Left thigh cms

Right thigh cms

Quad Measure. Measure 15cms up from the bottom of the knee cap and then measure around the leg.

Left quads cms

Right quads cms

E. Medical History

Do you have any of the following rheumatic diseases?

- ○ Sle/Lupus
- ○ Psoriatic arthritis
- ○ Ankylosing Spondyliis
- ○ Polymyalgia rheumatica
- ○ Fibromyalgia
- ○ Other
- ○ Gout
- ○ None of the above

Do you suffer, or have you suffered, from any of the following soft tissue rheumatic problems?

- ○ Frozen shoulder
- ○ Housemaids knee
- ○ Tennis elbow
- ○ Carpal tunnel syndrome
- ○ Other
- ○ None of the above

Have you any of the following operations?

Year of first operation

- ○ Hip replacement . . . . . .
- ○ Knee replacement . . . . . .
- ○ Arthoscopy . . . . . .
- ○ Other knee surgery . . . . . .
- ○ Elbow replacement . . . . . .
- ○ Shoulder replacement . . . . . .
- ○ Laminectomy . . . . . .

Draft

Study Type: T | Centre Code: S T H | Study No: | Visit Month:

F. Occupational History

FEMALE OCCUPATION

V1	V2	V3	I	II	IIIN	IIIM	IV	V
Housewife Student Unemployed	Retired	Permanently Disabled	University Lecturer Chartered Accountant Doctor Pharmacist Lawyer	Teacher Polytechnic Lecturer PR Librarian Nurse Social Worker	Secretary Clerical Receptionist Shop Assistant	Factory Skilled Day Nursery Cook	Waitress Usher Telephone Operator	Cleaner Kitchen Assistant Factory Work

MALE OCCUPATION

V1	V2	V3	I	II	IIIN	IIIM	IV	V
Divorced Single Dead	LongTerm Unemployed If unemployed less than 1 yr or retired record husband's longest occupation	Permanently Disabled	University Lecturer Chartered Accountant Doctor Pharmacist Lawer Chemist Clergy Surveyor Design	Teacher Polytechnic Lecturer Computer Prog Publican Manager	Shopworker Policeman Civil Servant Accountant Clerical	Caretaker Ambulance Skilled Builder	Assembler Packer Prison Officer Postman	Road Sweeper Dustman Porter

Present occupation (select one using the above as a guide)

V1 ○ V2 ○ V3 ○ I ○ II ○ IIIN ○ IIIM ○ IV ○ V ○

Years in present employment

Partners present occupation

V1 ○ V2 ○ V3 ○ I ○ II ○ IIIN ○ IIIM ○ IV ○ V ○

Enter as per coding using the twins own assessment of amount of knee bending (as a guide: typical clerical work would be classed as 'minimal', typical nursing duties would be classed as 'moderate', child rearing and farming would be classified as ' lot')

Knee Bending Score for Present Employment

○ None ○ Minimal ○ Moderate ○ Lot ○ All the Time/Continuously ○ N/A

Occupational History (continued)

Leave blank if no previous employment

Past occupation 1

V1 ○ V2 ○ V3 ○ I ○ II ○ IIIN ○ IIIM ○ IV ○ V ○

Years in this employment

Knee Bending Score for Past Employment 1

○ None ○ Minimal ○ Moderate ○ Lot ○ All the Time/Continuously ○ N/A

Past occupation 2

V1 ○ V2 ○ V3 ○ I ○ II ○ IIIN ○ IIIM ○ IV ○ V ○

Years in this employment

Knee Bending Score for Past Employment 2

○ None ○ Minimal ○ Moderate ○ Lot ○ All the Time/Continuously ○ N/A

Past occupation 3

V1 ○ V2 ○ V3 ○ I ○ II ○ IIIN ○ IIIM ○ IV ○ V ○

Years in this employment

Knee Bending Score for Past Employment 3

○ None ○ Minimal ○ Moderate ○ Lot ○ All the Time/Continuously ○ N/A

Study Type: T | Centre Code: S T H | Study No: _____ - _____ | Visit Month: _____

G. Exercise History

As a guide:
Ask about the twin's sporting activities, determine the time spent in each, and classify them using the tables, bearing in mind that vigorous activity should cause breathlessness or sweating, moderate activity should not. The classification of each sport may vary according to the history obtained from the twin.

	VIG-WB	MOD-WB	VIG-NWB	MOD-NWB
Swimming				X
Cycling				X
Running	X			
Keep fit		X		
Aerobics		X		
Tennis	X			
Badminton		X		
Squash	X			
Golf		X		
Skiing	X			
Ice Skating		X		
Bowls				Not!
Yoga				X
Dancing				X
Rugby	X			
Football	X			
Cricket	X			
Martial Arts		X		
Boxing	X			

Are you currently undertaking any regular physical activity once a week for at least one year? ○ No ○ Yes

If No, please go to question XX

How many minutes per week do you spend in vigorous weight-bearing activity? [ ][ ][ ][ ]

How many minutes per week do you spend in moderate weight bearing activity? [ ][ ][ ][ ]

How many minutes per week do you spend in vigorous non-weight bearing activity ? [ ][ ][ ][ ]

How many minutes per week do you spend in moderate non-weight bearing activity? [ ][ ][ ][ ]

At your Most Active Between 20 and 30, did you do at least 1 year of Regular Physical Activity ○ No ○ Yes ○ N/A

The year you did most regular physical activity between 20s and 30s [ ][ ][ ][ ]

How many minutes per week did you spend in vigorous sport in your 20s? [ ][ ][ ][ ]

How many minutes per week did you spend in moderate sport in your 20s? [ ][ ][ ][ ]

How many minutes per week did you spend in vigorous non-weight bearing activity in your 20s? [ ][ ][ ][ ]

How many actual hours per week do you spend on your feet at work and play? [ ][ ][ ]

How many hours at the most did you spend on your feet per week in your 20s? [ ][ ][ ]

H. Reproductive History

This section is for females only. If the twins are male, go to section I (Family Size)

H.1 How old were you when you had your first menstrual period? [ ][ ]

H.2 What is your menopausal status?

- ○ Definitely Pre-menopausal
- ○ Definitely Post-Menopausal
- ○ Probably Post-Menopausal if you have had a hysterectomy and are over* the age of 54
- ○ Possibly Post-Menopausal if you have had a hysterectomy and are aged* 50-54 years
- ○ Possibly Pre-menopausal if you had hysterectomy and are aged* 45-49 years
- ○ Probably Pre-Menopausal if your menopausal status is unknown and you are less than 45 years old

* or started HRT before the menopause
NOTE : take blood for FSH and OESTRADIOL if twin falls into any "probable" or "possible" category.

H.3 How old were you when you had your last regular period ? [ ][ ] years

H.4 What day of your menstrual cycle are you currently on? [ ][ ]

1 = 1st day of last period
Use -1 if HRT started before regular periods ceased

H.5 How many pregnancies have you had (including miscarriages, still births and terminations)? [ ][ ]

H.6 How many children were you involved in bringing up, including adopted? [ ][ ] (For foster children, 10 years fostering = 1 child equivalent)

H.7 Have you had a hysterectomy? ○ No ○ Yes ○ Partial ○ Don't Know

H.8 If yes, how old were you? [ ][ ] years

H.9 What was the main reason for the hysterectomy/endometrectomy?

- ○ Menorrhaghia (Excessive Menstruation)
- ○ Fibroids
- ○ Cancer
- ○ Other

H.10 Have you had any ovaries removed, with the hysterectomy or at any other time? ○ None ○ One ○ Both ○ Partial

H.11 Which of the following gynaecological operations have you had?

○ Dand C ○ Ovarian Cyst ○ Sterilization ○ Other ○ None

H.12 Have you ever taken the Oral Contraceptive Pill (OCP)?

○ No ○ Yes current ○ Yes, Ex-User ○ Don't Know

H.13 If yes, how old were you when you first went on to the OCP? [|] H.14 How long were you on the OCP? [|] months

H.15 Have you ever had Hormone Replacement Therapy (HRT)? ○ No ○ Yes, Current ○ Yes, Ex-User ○ Don't Know

H.16 If yes, how old were you when you first went on to HRT? [|]

H.17 How long were you on HRT? [|] months (If less than 1 month, enter as 1)

H.18 Enter the ROUTE and TYPE of HRT. Use the longest route if more than one type has been used.

HRT TYPE	HRT ROUTE	HRT PREPARATION
1 = Premarin	2	1
2 = Prempac C	2	2
3 = Estroderm TTS	1	1
4 = Nuvelle	2	1
5 = Hormonin	2	1
6 = Progynova	2	1
7 = Lival / Tibolone	2	3
8 = Other	n/a	n/a
9 = Don't Know	n/a	n/a
0 = n/a	n/a	n/a

Route

1) Patch ○ 2) Oral ○ 3) Vaginal ○ 4) N/A ○

Preparation

1) Oestrogen Only ○ 2) Oestrogen and Progesterone ○ 3) Oestrogen, Progesterone, Androgen ○ 4) Unspecified ○ N/A ○

Select one only

H.19 What was the main reason you began taking HRT?

○ Menopausal symptoms (flushing, sweating, palpitations)
○ Depression
○ Low Sex Drive
○ Worry about Osteoporosis/Thin Bones
○ To Improve General Health
○ Had a Hysterectomy
○ Ovaries had been removed
○ Tiredness
○ Headaches
○ Joint or Bone Pain
○ Twin did and she felt better
○ Advice of doctor
○ Other
○ N/A

H.20 Were there other reasons you began taking HRT?

○ Menopausal symptoms (flushing, sweating, palpitations)
○ Depression
○ Low Sex Drive
○ Worry about Osteoporosis/Thin Bones
○ To Improve General Health
○ Had a Hysterectomy
○ Ovaries had been removed
○ Tiredness
○ Headaches
○ Joint or Bone Pain
○ Twin did and she felt better
○ Advice of doctor
○ Other
○ N/A

Select one only

H.21 What was the main reason you stopped taking the HRT?

- ○ Did not find that the HRT helped the symptoms
- ○ Menopausal Symptoms came to and end
- ○ Advice of Doctor
- ○ Side effects - return of periods
- ○ Sore or swollen breasts
- ○ Nausea
- ○ Water Retention
- ○ Skin Rash or Irritation
- ○ Weight Gain
- ○ Twin Sister Stopped
- ○ Other
- ○ N/A

H.22 Were there any other reasons that you stopped taking the HRT?

- ○ Did not find that the HRT helped the symptoms
- ○ Menopausal symptoms came to an end
- ○ Advice of doctor
- ○ Side effects - return of periods
- ○ Sore or swollen breasts
- ○ Nausea
- ○ Water retention
- ○ Skin rash or irritation
- ○ Weight gain
- ○ Twin sister stopped
- ○ Other
- ○ N/A

H.23 Were / are your periods regular? (5 days difference or more = irregular) ○ No ○ Yes ○ Don't Know

H.24 If yes, how many days was your menstrual cycle (usually)? [ ][ ]

H.25 How many days did/do you bleed each cycle? [ ][ ]

H.26 Excluding pregnancies and the menopause, have you had any episodes where your periods stopped for more than six months?

○ No ○ Yes ○ Don't Know

H.27 If yes, how many months in total did you miss your period? [ ][ ][ ] months

H.28 How many children did you breast feed? [ ][ ]

H.29 How many months in total (for all children) did you breast feed? [ ][ ][ ] months

H.30 Do/did you get menopausal symptoms (e.g. sweating, flushing)? ○ No ○ Yes ○ N/A

H.31 Do/did you experience depression as a symptom of the menopause? ○ No ○ Yes ○ N/A

I. Family Size

This section is for male twins

I.1 How many children were you involved in bringing up (including adopted) [ ][ ] Foster children 10 years = 1

Twin Research Unit

GENERAL QUESTIONNAIRE - PART 2

2.0 PRODUCTION STH

To be completed by the research nurse

Study Type	Centre Code	Study No		Visit Month
T	STH			

Interviewer Code	Date (Day/Month/Year)
	/ / 19

J. Medical History

CVS disease

J.1 Have you had hypertension? ○ No ○ Yes ○ During pregnancy

J.2 Have you had Ischemic Heart Disease (including angina, heart attack)? ○ No ○ Yes

J.3 Have you ever lost the use of an arm, leg, your vision or ability to speak?

○ No ○ Yes (less than 24 hrs) ○ Yes (more than 24 hrs)

J.4 Can you walk a mile at your own pace on a flat level, not up or downhill, without stopping? ○ No ○ Yes

J.5 If not, what makes you stop?

○ Discomfort in calves ○ Discomfort in chest ○ Breathlessness ○ Other (i.e non cardiac reason) ○ N/A

J.6 Have you had coronary bypass surgery? ○ No ○ Yes

Pulmonary Disease Lung Function Measurements and Medication

J.7 Vitalograph machine number (if not taken leave blank) | | | |

J.8 Expected FEV1 - calculated using the lung function calculator | | . | |

(FEV = Forced Expiration Volume)

J.9 Number of blows into the vitalograph | |

J.10 Measured FEV1 - recorded from x axis at 1 sec. | | . | | |

The highest recording is taken (If between 2 lines estimate downwards)

J.11 Measured FVC - recorded from x axis at 6 second. (if between 2 lines estimate downwards) | | . | | |

(FVC = Forced Volume Capacity)

J.12 Room Temperature | | degrees c.

J.13 In the past 2 weeks, have you had a cold or 'flu-like illness'? ○ No ○ Yes

J.14 Have you ever had asthma? ○ No ○ Yes (whether treated with medication or not)

J.15 If yes, what medication are you currently taking for it?

○ Steroidal (blue) ○ Non-steroidal (brown) ○ Other

J.16 How often are you taking medication for asthma?

○ PRN (less than once a day) ○ OD ○ BD ○ TDS ○ QDS ○ PRN (more than QDS)

J.17 Time since taken | | . | |

hrs mins

K. Hair and Teeth

K.1 Number of second or adult teeth lost (through decay)

☐ 0 ☐ 1-5 ☐ 6-10 ☐ 11-20 ☐ over 20

K.2 Age you lost all second or adult teeth ____

K.3 Original young adult hair colour

Select one

☐ Brown

☐ Black

☐ Blonde

☐ Red

☐ Auburn (chestnut)

☐ Strawberry blonde (reddish-fair)

☐ Light/brown/fair/mousey

☐ Other

K.4 Age you noticed more than one white hair ____

K.5 Age at which you turned mostly white ____

K.6 Age you turned completely white ____

L. Fracture History and Osteoporosis

Complete this section if # bone. If no #, go to section M

Site of fracture 1	Site of fracture 2	Site of fracture 3	Site of fracture 4	Site of fracture 5
☐ spine	☐ spine	☐ spine	☐ spine	☐ spine
☐ wrist	☐ wrist	☐ wrist	☐ wrist	☐ wrist
☐ hip	☐ hip	☐ hip	☐ hip	☐ hip
☐ arm not wrist	☐ arm not wrist	☐ arm not wrist	☐ arm not wrist	☐ arm not wrist
☐ ankle	☐ ankle	☐ ankle	☐ ankle	☐ ankle
☐ leg not ankle	☐ leg not ankle	☐ leg not ankle	☐ leg not ankle	☐ leg not ankle
Severity of injury 1	Severity of injury 2	Severity of injury 3	Severity of injury 4	Severity of injury 5
☐ Major injury (RTA)	☐ Major injury (RTA)	☐ Major injury (RTA)	☐ Major injury (RTA)	☐ Major injury (RTA)
☐ Minor injury fall < ht of chair	☐ Minor injury fall < ht of chair	☐ Minor injury fall < ht of chair	☐ Minor injury fall < ht of chair	☐ Minor injury fall < ht of chair
Age of fracture 1 ☐☐ yrs	Age of fracture 2 ☐☐ yrs	Age of fracture 3 ☐☐ yrs	Age of fracture 4 ☐☐ yrs	Age of fracture 5 ☐☐ yrs

Have you been diagnosed as having osteoporosis? ☐ No ☐ Yes ☐ Don't know

If yes, what treatment did/do you have?

☐ No treatment

☐ Don't know

☐ HRT

☐ Bisphosphonates (Didronel or Fosamax)

☐ Calcium and Vitamin D

☐ Other treatments

Draft

Study Type: T | Centre Code: S T H | Study No: _____ - | | | Visit Month: | |

Diabetes

Do you have diabetes?

☐ No

☐ Yes, insulin dependent Type 1, treated with insulin from diagnosis

☐ Yes, non insulin Type 2

☐ Have/had diabetes unsure what type

☐ Gestational diabetes

Other Endocrine

Have you ever had thyroid disease? ☐ No ☐ Yes Hypothyroid or Hyperthyroid disease ☐ Don't know

Cancer

Have you had:

Breast Cancer?	Colon Polyps?	Colon Cancer?	Gastrectomy?
☐ No	☐ No	☐ No	☐ No
☐ Yes	☐ Yes	☐ Yes	☐ Yes
			☐ Partial

Raynaud's Phenomenon

	Hands	Feet
Have the following ever or frequently(at least once a week) changed colour in the cold weather?	☐ Yes ☐ No	☐ Yes ☐ No
If yes, at what age did this begin?	☐☐ yrs	☐☐ yrs
Is this still a problem?	☐ Yes ☐ No	☐ Yes ☐ No
If not, (i.e not happened within the last 12 mths) at what age did it end?	☐☐ yrs	☐☐ yrs
Do you remember a sequence of colour changes in your hands or feet? If colour changes included/white/blue-purple then score. If not, cross none.	☐ None ☐ One colour ☐ Two colours ☐ More than two colours	☐ None ☐ One colour ☐ Two colours ☐ More than two colour
Is the colour change accompanied by numbness?	☐ Yes ☐ No	☐ Yes ☐ No
Is the colour change accompanied by pain?	☐ Yes ☐ No	☐ Yes ☐ No

Does anyone else in the family (other than your twin) notice these symptoms?

Parents

☐ No ☐ Yes ☐ Don't know

Other sib

☐ No ☐ Yes ☐ Don't know / N/A

Children

☐ No ☐ Yes ☐ Don't know / N/A

Joint disease

Have you ever been diagnosed as having Arthritis of the knee(s)? ☐ No ☐ Yes ☐ Don't know

Have you ever been diagnosed as having Arthritis of the Hip(s)? ☐ No ☐ Yes ☐ Don't know

Have you ever been diagnosed as having Arthritis of the Hand(s)? ☐ No ☐ Yes ☐ Don't know

Do you have Rheumatoid Arthritis?

☐ No ☐ Yes (Confirmed by blood test) ☐ Yes (Not confirmed by blood test) ☐ Don't know

If yes, at what age did this begin? ☐☐ years

Have you ever had pain or stiffness in the following joints?

Left hand	Right hand	Left Knee	Right knee	Left hip	Right hip	Back	Neck	Other
☐ No	☐ No	☐ No	☐ No	☐ No	☐ No	☐ No	☐ No	☐ No
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes

Age the pain or stiffness stated in these joints

Left hand	Right hand	Left knee	Right knee	Left hip	Right hip	Back	Neck	Other
☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐

Duration of pain in the following joints

Left hand	Right hand	Left knee	Right knee	Left hip	Right hip	Back	Neck	Other
☐ < 5 days	☐ < 5 days	☐ < 5 days	☐ < 5 days	☐ < 5 days	☐ < 5 days	☐ < 5 days	☐ < 5 days	☐ < 5
☐ 5-15 days	☐ 5-15 days	☐ 5-15 days	☐ 5-15 days	☐ 5-15 days	☐ 5-15 days	☐ 5-15 days	☐ 5-15 days	☐ 5-15
☐ 16-30 days	☐ 16-30 days	☐ 16-30 days	☐ 16-30 days	☐ 16-30 days	☐ 16-30 days	☐ 16-30 days	☐ 16-30 days	☐ 16-30
☐ > 30 days	☐ > 30 days	☐ > 30 days	☐ > 30 days	☐ > 30 days	☐ > 30 days	☐ > 30 days	☐ > 30 days	☐ > 30

Have you had pain in the last month in the following joints?

Left hand	Right hand	Left Knee	Right knee	Left hip	Right hip	Back	Neck	Other
☐ No	☐ No	☐ No	☐ No	☐ No	☐ No	☐ No	☐ No	☐ No
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes

Number of full days pain lasted in the following joints in the last month (refers to continuous pain from first thing in the morning to the last thing at night).If < 1 day enter 0 or enter number of days.

Left hand	Right hand	Left knee	Right knee	Left hip	Right hip	Back	Neck	Other
☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐	☐☐

Is the pain in the following joints in the last year better, worse or same

Left hand	Right hand	Left knee	Right knee	Left hip	Right hip	Back	Neck	Other
☐ Better	☐ Better	☐ Better	☐ Better	☐ Better	☐ Better	☐ Better	☐ Better	☐ Better
☐ Same	☐ Same	☐ Same	☐ Same	☐ Same	☐ Same	☐ Same	☐ Same	☐ Same
☐ Worse	☐ Worse	☐ Worse	☐ Worse	☐ Worse	☐ Worse	☐ Worse	☐ Worse	☐ Worse

How many painful episodes have you had in the last year in the following joints?

Left hand	Right hand	Left knee	Right knee	Left hip	Right hip	Back	Neck	Other
☐ None	☐ None	☐ None	☐ None	☐ None	☐ None	☐ None	☐ None	☐ None
☐ One	☐ One	☐ One	☐ One	☐ One	☐ One	☐ One	☐ One	☐ One
☐ 2 - 4	☐ 2 - 4	☐ 2 - 4	☐ 2 - 4	☐ 2 - 4	☐ 2 - 4	☐ 2 - 4	☐ 2 - 4	☐ 2 - 4
☐ > 5	☐ > 5	☐ > 5	☐ > 5	☐ > 5	☐ > 5	☐ > 5	☐ > 5	☐ > 5

Have you ever injured the following joints long enough to rest it for more than a week?

Left hand	Right hand	Left Knee	Right knee	Left hip	Right hip	Back	Neck	Other
☐ No	☐ No	☐ No	☐ No	☐ No	☐ No	☐ No	☐ No	☐ No
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes

Draft

Study Type: T | Centre Code: STH | Study No: ____ - ____ | Visit Month: ____

Do you get early morning stiffness in the following joints ? If yes, give the length of time (in minutes) it lasts

Left Hand	☐ No ☐ Yes (every morning) ☐ 1/2 morn Y & 1/2 N	____ mins
Right Hand	☐ No ☐ Yes (every morning) ☐ 1/2 morn Y & 1/2 N	____ mins
Left Knee	☐ No ☐ Yes (every morning) ☐ 1/2 morn Y & 1/2 N	____ mins
Right Knee	☐ No ☐ Yes (every morning) ☐ 1/2 morn Y & 1/2 N	____ mins
Left Hip	☐ No ☐ Yes (every morning) ☐ 1/2 morn Y & 1/2 N	____ mins
Right Hip	☐ No ☐ Yes (every morning) ☐ 1/2 morn Y & 1/2 N	____ mins
Back	☐ No ☐ Yes (every morning) ☐ 1/2 morn Y & 1/2 N	____ mins
Neck	☐ No ☐ Yes (every morning) ☐ 1/2 morn Y & 1/2 N	____ mins
Other	☐ No ☐ Yes (every morning) ☐ 1/2 morn Y & 1/2 N	____ mins

Joint Examination

Is there definite evidence of boney artiocular swelling affecting any of the DIP or PIP joints? ○ Yes ○ No

M. Drug History (See coding sheet)

	Current Drug Code	Age started	Duration (months)
1)	☐☐.☐	☐☐	☐☐☐
2)	☐☐.☐	☐☐	☐☐☐
3)	☐☐.☐	☐☐	☐☐☐
4)	☐☐.☐	☐☐	☐☐☐
5)	☐☐.☐	☐☐	☐☐☐
6)	☐☐.☐	☐☐	☐☐☐
7)	☐☐.☐	☐☐	☐☐☐
8)	☐☐.☐	☐☐	☐☐☐
9)	☐☐.☐	☐☐	☐☐☐
10)	☐☐.☐	☐☐	☐☐☐

	Past Drug Code	Age started	Duration (months)
1)	☐☐.☐	☐☐	☐☐☐
2)	☐☐.☐	☐☐	☐☐☐
3)	☐☐.☐	☐☐	☐☐☐
4)	☐☐.☐	☐☐	☐☐☐
5)	☐☐.☐	☐☐	☐☐☐
6)	☐☐.☐	☐☐	☐☐☐
7)	☐☐.☐	☐☐	☐☐☐
8)	☐☐.☐	☐☐	☐☐☐
9)	☐☐.☐	☐☐	☐☐☐
10)	☐☐.☐	☐☐	☐☐☐

N. Tobacco and Alcohol

N.1 Have you ever smoked cigarettes, cigars or a pipe?

☐ Never smoked

☐ Yes, current smoker

☐ Yes ex-smoker

If you "Never Smoked", please go to question N.7
If you are a "Current" or "Ex" smoker, continue with question N.2

N.2 At what age did you start smoking? ☐☐

N.3 Do you smoke at all nowadays? ☐ Yes ☐ No

N.4 If ex-smoker at what age did you stop altogether? ☐☐

N.5 How many cigarettes do/did you usually smoke each day? ☐☐☐

N.6 What is your Pack Year habit? ☐☐ (No. cigarettes per day smoked X No. years smoked) / 20

N.7 What is your current average alcohol consumption?

☐ Never
☐ Social Occasions Only
☐ 1-5 Units per week
☐ 6-10 Units per week
☐ 11-15 Units per week
☐ 16-20 Units per week
☐ 21-40 Units per week
☐ More than 40 units per week
☐ Amount Unspecified

N.8 What is your lifetime average alcohol consumption?

☐ Never
☐ Social Occasions Only
☐ 1-5 Units per week
☐ 6-10 Units per week
☐ 11-15 Units per week
☐ 16-20 Units per week
☐ 21-40 Units per week
☐ More than 40 units per week
☐ Amount Unspecified

O. About Your Relatives

Where information is unavailable, please leave blank.

Your Mother

Year of birth | Year of death | Country of birth

Your Father

Year of birth | Year of death | Country of birth

Your Grandparents

Maternal grandmother's country of birth

Maternal grandfather's country of birth

Paternal grandmother's country of birth

Paternal grandfather's country of birth

Your Children

Please give, in birth order, including stillborn or premature babies that died soon after being born, your children's year of birth and, if applicable, year of death

	Year of birth	Year of death	Sex
1			○ Male ○ Female ○ Unknown
2			○ Male ○ Female ○ Unknown
3			○ Male ○ Female ○ Unknown
4			○ Male ○ Female ○ Unknown
5			○ Male ○ Female ○ Unknown
6			○ Male ○ Female ○ Unknown
7			○ Male ○ Female ○ Unknown
8			○ Male ○ Female ○ Unknown
9			○ Male ○ Female ○ Unknown
10			○ Male ○ Female ○ Unknown

Study Type	Centre Code	Study No		Visit Month
T	S T H		-	

Your Brothers and Sisters

Please state, in birth order, your siblings year of birth and, if applicable, year of death

	Year of birth	Year of death	Sex
1			○ Male ○ Female ○ Unknown
2			○ Male ○ Female ○ Unknown
3			○ Male ○ Female ○ Unknown
4			○ Male ○ Female ○ Unknown
5			○ Male ○ Female ○ Unknown
6			○ Male ○ Female ○ Unknown
7			○ Male ○ Female ○ Unknown
8			○ Male ○ Female ○ Unknown
9			○ Male ○ Female ○ Unknown
10			○ Male ○ Female ○ Unknown

T S T H

Sites of Fracture

E.g of a major injury include a road traffic accident and minor injuries - a fall from lower than chair height

Mother Age at fracture

Severity

- ☐ Spine ☐ Major ☐ Minor ☐ Don't know
- ☐ Wrist ☐ Major ☐ Minor ☐ Don't know
- ☐ Hip ☐ Major ☐ Minor ☐ Don't know
- ☐ Arm (not wrist) ☐ Major ☐ Minor ☐ Don't know
- ☐ Ankle ☐ Major ☐ Minor ☐ Don't know
- ☐ Leg ☐ Major ☐ Minor ☐ Don't know
- ☐ Don't know

Father Age at fracture

Severity

- ☐ Spine ☐ Major ☐ Minor ☐ Don't know
- ☐ Wrist ☐ Major ☐ Minor ☐ Don't know
- ☐ Hip ☐ Major ☐ Minor ☐ Don't know
- ☐ Arm (not wrist) ☐ Major ☐ Minor ☐ Don't know
- ☐ Ankle ☐ Major ☐ Minor ☐ Don't know
- ☐ Leg ☐ Major ☐ Minor ☐ Don't know
- ☐ Don't know

Any sister Age at fracture

Severity

- ☐ Spine ☐ Major ☐ Minor ☐ Don't know
- ☐ Wrist ☐ Major ☐ Minor ☐ Don't know
- ☐ Hip ☐ Major ☐ Minor ☐ Don't know
- ☐ Arm (not wrist) ☐ Major ☐ Minor ☐ Don't know
- ☐ Ankle ☐ Major ☐ Minor ☐ Don't know
- ☐ Leg ☐ Major ☐ Minor ☐ Don't know
- ☐ Don't know
- ☐ Not applicable

Any brother Age at fracture

Severity

- ☐ Spine ☐ Major ☐ Minor ☐ Don't know
- ☐ Wrist ☐ Major ☐ Minor ☐ Don't know
- ☐ Hip ☐ Major ☐ Minor ☐ Don't know
- ☐ Arm (not wrist) ☐ Major ☐ Minor ☐ Don't know
- ☐ Ankle ☐ Major ☐ Minor ☐ Don't know
- ☐ Leg ☐ Major ☐ Minor ☐ Don't know
- ☐ Don't know
- ☐ Not applicable

Any children Age at fracture

Severity

- ☐ Spine ☐ Major ☐ Minor ☐ Don't know
- ☐ Wrist ☐ Major ☐ Minor ☐ Don't know
- ☐ Hip ☐ Major ☐ Minor ☐ Don't know
- ☐ Arm (not wrist) ☐ Major ☐ Minor ☐ Don't know
- ☐ Ankle ☐ Major ☐ Minor ☐ Don't know
- ☐ Leg ☐ Major ☐ Minor ☐ Don't know
- ☐ Don't know
- ☐ Not applicable

Does anyone in your family have osteoporosis?

MOTHER	FATHER	ANY SISTER	ANY BROTHER	ANY CHILD
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know

If yes, what treatment was used? Examples of Bisphosphanate are Didronel and Fosamax

☐ No Treatment	☐ No Treatment	☐ No Treatment	☐ No Treatment	☐ No Treatment
☐ HRT	☐ HRT	☐ HRT	☐ HRT	☐ HRT
☐ Bisphosphanate	☐ Bisphosphanate	☐ Bisphosphanate	☐ Bisphosphanate	☐ Bisphosphanate
☐ Calcium and Vit D	☐ Calcium and Vit D	☐ Calcium and Vit D	☐ Calcium and Vit D	☐ Calcium and Vit D
☐ Other	☐ Other	☐ Other	☐ Other	☐ Other
☐ Don't know	☐ Don't know	☐ Don't know	☐ Don't know	☐ Don't Know

Does anyone in the family have Arthritis of the knee?

☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know

Does anyone in your family have Arthritis of the hip?

☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know

Does anyone in your family have Arthritis of the hands, including wrists?

☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know

Does anyone in your family have Thyroid Disease?

☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know

Does anyone in you family have Rheumatoid Arthritis?

☐ Yes (confirmed by blood test) ☐ Yes (not confirmed by blood test) ☐ No ☐ Don't Know	☐ Yes (confirmed by blood test) ☐ Yes (not confirmed by blood test) ☐ No ☐ Don't Know	☐ Yes (confirmed by blood test) ☐ Yes (not confirmed by blood test) ☐ No ☐ Don't Know	☐ Yes (confirmed by blood test) ☐ Yes (not confirmed by blood test) ☐ No ☐ Don't Know	☐ Yes (confirmed by blood test) ☐ Yes (not confirmed by blood test) ☐ No ☐ Don't Know

Draft

Study Type: T | Centre Code: S T H | Study No: _____ - _____ | Visit Month: _____

MOTHER	FATHER	ANY SISTER	ANY BROTHER	ANY CHILD
Does anyone in your family have insulin Dependent Diabetes Mellitus Type 1?			Treated with Insulin from diagnosis	
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know
☐ Yes (unsure what type)	☐ Yes (unsure what type)	☐ Yes (unsure what type)	☐ Yes (unsure what type)	☐ Yes (unsure what type)

Does anyone in your family have non insulin Dependent Diabetes Mellitus Type 2?			Treated by diet/tablet sat the beginning may have had to progress on to insulin	
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know
☐ Yes (unsure what type)	☐ Yes (unsure what type)	☐ Yes (unsure what type)	☐ Yes (unsure what type)	☐ Yes (unsure what type)

Does anyone in you family have Gestational Diabetes?				
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know
☐ Yes (unsure what type)	☐ Yes (unsure what type)	☐ Yes (unsure what type)	☐ Yes (unsure what type)	☐ Yes (unsure what type)

Does anyone in your family suffer from Hypertension?				
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know

Does anyone in your family have Ischemic Heart Disease (including angina, Heart attack)?				
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know

Has anyone in your family had a stroke?				
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know

Does anyone in your family have Asthma (however treated)?				
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know

Draft

Study Type: T | Centre Code: STH | Study No: _____ - _____ | Visit Month: _____

MOTHER	FATHER	ANY SISTER	ANY BROTHER	ANY CHILD
Does/did your mother, any sister or any child have/had Breast Cancer?				
☐ Yes		☐ Yes		☐ Yes
☐ No		☐ No		☐ No
☐ Don't Know		☐ Don't Know		☐ Don't Know
Does anyone in your family have Colon Cancer?				
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know
Does anyone in your family have Gasterectomy?				
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know
Does anyone in your family have Cholecystectomy?				
☐ Yes	☐ Yes	☐ Yes	☐ Yes	☐ Yes
☐ No	☐ No	☐ No	☐ No	☐ No
☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know	☐ Don't Know

APPENDIX B

CHINGFORD STUDY QUESTIONNAIRE

OSTEOPOROSIS / OSTEOARTHRITIS CLINIC Joint questionnaire

Name [redacted] Date Number 1

Miss/Mrs/Ms

Address Hospital No. 2

Telephone _____ Home _____ Work

Date of Birth (DDMMYY) 3

Age now 4

GP Name _____

GP address _____

Occupation 5

Previous occupation _____

Husband occupation 6

Hand dominance (1=left 2=right 3=both) 7

(for all coding 1=yes 0=no -1=missing)

Reproductive History

Age menarche 8

Age menopause (01=menstruating) 9

Para 10 11

Hysterectomy 12

age 13

indication (1=menorrhagia 2=fibroids 3=cancer 4=other) 14

Ovaries removed 0=none 1= one 2= both 15

Gynae ops 16
0=none
1=D&C
2=ov. cyst
3=steri
4=other

OCP 17

Age 18

Duration(mmm) 19

HRT 20

Age 21

Duration(mmm) 22

Type: _____

Medical History

Current medication? 23
Type: _____

Blood pressure 24
Date: _____

Diuretics 25
Date: _____

Diabetes 26
Date: _____

Thyroid 27
Date: _____

Ortho ops 28
Date: _____

Fractures(10yrs) 29
Date: _____ 30
Type: _____ 31

Medical History cont.

Weight (kg)
Max 32 ☐

Min 33 ☐

Age 20 34 ☐

Adult height 35 ☐

Smoking
0=never
1=current
2=ex(how long stopped ____ yrs) 36 ☐

Max (per day) 37 ☐

Length (yrs) 38 ☐

Alcohol 39 ☐
0=never
1=weekly
2=social occ.
Units (per wk) 40 ☐

Any operations or serious illness?

Ca. 41 ☐
Date: _____

CVD 42 ☐
Date: _____

Gastrectomy 43 ☐
Date: _____

Cholecystectomy 44 ☐
Date: _____

Other
Date: _____

Family History

Arthritis
0=none
1=probable
2=definite
3=adopted
(+ age onset)

	Hand	Knee
Mother	☐	☐
MGM	☐	☐
Aunt	☐	☐
Father	☐	☐
Sister	☐	☐
Brother	☐	☐
Children	☐	☐

Obesity

Mother ☐

Father ☐

JOINTS AFFECTED BY ARTHRITIS

JOINT	YEAR	SYMPTOMS (also state if present)
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

Name and address of contact: _____

_____

_____

_____

_____

_____

Relationship to patient: _____

BACK PAIN/RISK FACTORS FOR SPINAL FRACTURES (for all answers 1=Yes 0=No)

Name _____ Number _____ Date _____

1. Have you ever had back pain lasting more than a week?

☐ When/dates? _____

What problem? _____

2. Have you ever seen a Dr. about back pain?

☐ What treatment did you have? _____

3. Have you noticed a loss of height, a stoop or a hump?

☐ How much height have you lost? ☐ inches.

4. Have you ever taken steroid tablets?

☐ Dates _____ How long for? ☐ mths.

5. Have you ever taken calcium tablets?

☐ Dates _____ How long for? ☐ mths. Dose ☐ mg

6. How many pints of milk do you drink in a week? ☐

7. How many times a week do you eat hard cheese? ☐

8. How many small pots of yoghurt do you eat a week? ☐

☐ mg Calcium intake per week please leave

9. Have any of the following relatives had: (1=yes 0=No)

Fracture of:	Mother	Grandmother(mothers side)	Sister	Aunt(mothers side)
Hip	☐	☐	☐	☐
Wrist	☐	☐	☐	☐
Spine	☐	☐	☐	☐

(add number next to box for amount of times fractured)

10. Do any of the following relatives have: (1=Yes 0=No)

	Mother	Grandmother(mothers side)	Sister	Aunt(mothers side)
lost height	☐	☐	☐	☐
(add no. of inches lost next to box)				
Stoop	☐	☐	☐	☐
Hump	☐	☐	☐	☐